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THE EFFECT OF SULFUR DIOXIDE AND COLD STRESS ON CATTLE

by

LIOUDMILA KOMARNISKY

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Nutrition and Metabolism

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

THE EFFECT OF SULFUR DIOXIDE AND COLD STRESS ON CATTLE

submitted by **LIUODMILA KOMARNISKY**

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY in **NUTRITION AND METABOLISM**.

Dated August 8, 2003

Alma Kennedy

This work is dedicated

to my foster parents Fatima and Muhamed-Ali

and to my dear sister Roza

for their unconditional love, support and encouragement

ABSTRACT

The objectives of this investigation were to test the hypotheses that exposure of cattle to air with sulfur dioxide (SO_2) would: 1) suppress immune cell activity; 2) induce pulmonary cell damage; 3) increase energy metabolism; and 4) that cold stress influences the toxicity of SO_2 in cattle. The effect of SO_2 on beef cattle was examined in thermo-neutral (warm) and cold environments. Steers were exposed in a head-only inhalation system to either fresh air (control) or to air containing 1, 5, and 20 ppm of SO_2. Exposure of steers to 5 and 20 ppm of SO_2 increased energy expenditure by 20 – 27% ($P < 0.05$, $n = 6$) in a warm environment. SO_2 at 1, 5 and 20 ppm increased energy expenditure by 28, 39 and 45 %, respectively ($P < 0.05$, $n = 4$) in the cold environment. Exposure of steers to 1, 5 and 20 ppm of SO_2 reduced the proportion of $CD4^+$ cells, increased the proportion of $CD8^+$ cells and increased the proportion of $CD14^+$ cells in peripheral blood ($P < 0.05$) in both environments. Exposure to 1, 5 and 20 ppm of SO_2 increased the ratio of damaged to normal pulmonary cells ($P< 0.05$), increased activity of the lactate dehydrogenase and alkaline phosphatase ($P < 0.05$), reduced the proportion of macrophages ($P< 0.005$) and increased the proportion of neutrophils ($P< 0.05$) in broncho-alveolar lavage fluids of steers. The respiratory response of peripheral blood neutrophils to stimulation was significantly decreased by exposure of steers to 1, 5, and 20 ppm of SO_2 ($P < 0.05$, and $P < 0.01$) in the warm environment. Supplementation with a single injection of vitamin E and selenium improved plasma vitamin E ($P < 0.05$) and selenium status ($P < 0.05$) during the two-week period after the end of SO_2 exposure and increased the number of blood neutrophils ($P < 0.05$).

The increased energy expenditure, decreases in vitamin E, and reductions in neutrophil respiratory activity and nitric oxide production by alveolar macrophages in response to SO_2 are expected to have negative effects on productivity and health of cattle.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ALP	alkaline phosphatase
APP	acute phase proteins
ATP	adenosine triphosphate
BAL	broncho-alveolar lavage
BSA	bovine serum albumin
BW	body weight
CBC	complete blood count
CCM	complete culture media
CP	crude protein
DHR	dihydrorhodamine
DMSO	dimethyl sulfoxide
FCS	fetal calf serum
FITCI	fluoroscein isothiocyanate
FSC	forward scatter
Hpt	haptoglobins
HP	heat production
IL	interleukin
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
NO	nitric oxide
PBS	phosphate buffered saline
PE	phycoerythrin
RBC	red blood cells
PMA	phorbol myristate acetate
RIA	radioimmunoassay
SEM	standard error of the mean
UV	ultraviolet
WBC	white blood cells

GENERAL INTRODUCTION

Sulfur dioxide (SO_2) is a major air pollutant in Alberta, in Canada and worldwide (Alberta's Clean Air Act 1985). During cold spells, ground level concentrations of SO_2 increase and cattle exposures may occur. Epidemiologic studies on air pollution have shown an association between air pollution linked illness and cold weather (Webster 1981; Keatinge and Donaldson 2001). During cold spells, decreased air quality may be due to decreased dispersion of pollutants, return of pollutants to the surface and a decline in ground level mixing of air and thermal inversions (Goldstein et al 1982). In humans increased physical activity has been shown to increase sensitivity to air pollutants because of increases in respiratory ventilation associated with higher rates of metabolism and heat production (Duggan and Haisman 1992). By analogy increased metabolism in cold stressed cattle may increase sensitivity to SO_2.

Cold weather is a stressor for cattle and increases energy metabolism (Scott *et al* 1993). In young calves on a fixed feed intake, cold exposure resulted in a 20 % increase in heat production, and reduced whole body protein growth by about 46% in association with significant reductions in protein synthesis in skeletal muscle, kidney and skin (Scott *et al* 1993). Increasing feed intake by 20% in the cold environment restored protein growth and tissue protein synthesis rates to levels approaching those in the warm environment, although feed efficiency for growth was reduced by cold stress (Scott *et al* 1993). Acute cold stress can also influence other physiological functions and activity and numbers of cells of the immune system (Sundaresan et al 1990).

There have been no detailed toxicology and physiological studies done on SO_2 exposure in cattle. Sulfur dioxide has pronounced irritant effects on the respiratory system in laboratory animals and causes bronchial constriction and reduced particle clearance from lung of donkeys (Spiegelman et al 1968). Effects of SO_2 on the immune system of animals have not been determined, although other stressors have been shown to alter immune function (Zelikoff et al 1994). In spite of the natural occurrence of cold weather and coincidental accidental exposure to air pollution containing SO_2, the interactions between these environmental factors have not been studied in cattle.

Effects of the combined stressors may be additive or synergistic leading to increased susceptibility to respiratory infection. Bronchoconstictor effects of SO_2 may

compromise metabolic responses to cold stress and reduce the cold tolerance of cattle. Energy and nutrient intake of cattle may modify the responses of cattle to combinations of cold and SO_2 exposure and provision of supplemental energy or specific nutrients may be useful in providing therapy to affected animals.

This research aimed to increase knowledge of the factors which influence the severity of response of cattle to environmental pollutants that are commonly encountered in Alberta and other regions of Canada. This information will help in the establishment of guidelines for dealing with air pollutants to improve health and safety of cattle. By identifying the range of immunological, metabolic and physiological responses, it may be possible to suggest strategies for treatment of animals showing clinical symptoms of toxicity. There may be nutritional strategies identified that will be effective in reducing the impact of environmental pollutants on health of cattle. The obtained information may also form a basis for risk assessment programs.

Since there was no data available on dose-responses of cattle to known levels of SO_2, an initial experiment (experiment 1) was conducted with 2 steers to establish dose rates of SO_2 that would exceed threshold levels for induction of toxic, but not lethal, reactions in a thermo-neutral environment. The experiment began with an examination of doses of SO_2 that mimic anticipated environmental exposure levels at a workplace for humans (5 ppm) (ACGIH 1996) and a higher level of SO_2 (20 ppm), that may provoke immunological, metabolic or physiological responses of cattle to intoxication. This experiment also provided information on extent and rate of recovery following SO_2 exposure. Experiments 2 and 3 employed larger numbers of animals and were designed to use the levels of SO_2 that have been tested in experiment 1 (5 and 20 ppm) and the lower level of 1 ppm of SO_2, which may be more representative of SO_2 levels near accidental emission sources. Experiments 1 and 2 were conducted in a thermo-neutral environment, and experiment 3 examined exposure to SO_2 in both thermo-neutral and cold environments (designated as "warm" and "cold"). Experiment 4 was a follow-up to experiments 2 and 3 where steers previously exposed to 20 ppm of SO_2 were given an intramuscular injection of vitamin E and selenium (Se) treatment and compared to cattle with similar exposure without vitamin E/ selenium treatment. Please, refer to the diagrams illustrating the designs of the experiments 1, 2, 3, and 4, which indicate

numbers of animals used in each experiment, the SO_2 exposure levels, and the number of exposure days (Figures 1, 2, and 3).

The respiratory system is the most studied area of the effect of SO_2 in humans and laboratory animals, and to our knowledge, there is very limited information on the systemic effects of SO_2, not only in cattle, but in humans and other animals as well. Therefore, a number of systemic physiological, immunological and toxicological parameters were selected to test during the SO_2 trials. The chapters of the thesis are arranged in a paper format and include groups of related parameters which were analyzed throughout the course of all experiments.

Chapter 1 is a literature review on the topic of air pollution involving SO_2. It describes the known effects of SO_2 on humans and animals, gives an introduction into the respiratory system as a route for inhalation of air pollutants, the distribution and metabolism of SO_2 in the body, and relation of SO_2 with other air pollutants. It also includes a discussion of production of free radicals caused by SO_2 exposure and how human and animal organisms deal with these. The chapter also reviews literature on factors affecting the immune system, metabolic rate and other physiological parameters. A brief description of the immune system and the use of bovine monoclonal antibodies in immunoassays are also presented. The roles of vitamin E, vitamin A and selenium in detoxification of the organism from the deleterious effects of free radicals are included. The discussions were related to cattle where it was possible to find appropriate information.

The main general hypotheses addressed by this research study are that the breathing of air with SO_2 1) will suppress immune cell function, 2) will increase metabolic rate of the affected animals, 3) will have deleterious effect on the function of the respiratory system in cattle, and 4) that cold stress will exacerbate physiological, such as heart rate, respiratory frequency and rectal temperature, responses to SO_2.

Specific hypotheses include the following:

Neutrophil respiratory activity will be decreased by SO_2 inhalation due to impact on the functional ability of the neutrophils.

Plasma concentrations of vitamins A and E and serum concentrations of selenium will be decreased due to consumption of these antioxidants in the detoxifying process of free radicals.

The concentration of circulatory acute phase proteins will increase as a result of the inflammation of the pulmonary tissue;

SO_2 inhalation will increase the destruction of pulmonary cells as a result of direct exposure on pulmonary system;

The production of nitric oxide by alveolar macrophages will increase as a result of stimulation of macrophages by direct exposure to SO_2 in the lungs of cattle;

The activity of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) in broncho-alveolar (BAL) fluids will increase as a consequence of damage to pulmonary cells;

The ratio of T helper cells (CD4) to cytotoxic T cells (CD8) will change as a result of the changes in immune system function produced by SO_2 inhalation;

The number of proinflammatory cells in blood and activity of enzymes in blood serum will increase due to inflammation and stress;

The metabolic rate in cattle exposed to SO_2 will be increased as a result of overall stress effect.

To test these hypotheses we used a head-only inhalation exposure system for cattle, the function of which is described in detail in Chapter 2. The SO_2 inhalation system for cattle allowed accurate delivery of air with SO_2 simultaneously into 4 hoods.

The remaining chapters focus on the following main aspects of the study:

Chapter 3 Effects of SO_2 on neutrophil respiratory activity;

Chapter 4. Plasma concentration of vitamin E, vitamin A and serum concentrations of selenium and acute phase proteins;

Chapter 5. Differential count of pulmonary cells and nitric oxide production by pulmonary macrophages;

Chapter 6. Mononuclear leukocyte subtypes in response to SO_2;

Chapter 7. Energy metabolism and related physiological parameters;

Chapter 8 is a summary of the entire thesis and general discussion.

This study was planned and designed to achieve a specific goal to determine whether or not a difference occurs due to treatment (exposure to SO_2 or "cold" stress), proposed by Rysin (1985) for clinical research in inhalation toxicology. To our knowledge, the research presented in this thesis is the first comprehensive study in cattle to document changes in metabolic rate, immune cell function, and pulmonary cell status as a result of inhalation of air containing control concentrations of SO_2.In this study, a control group of steers was compared to a treated group and tested the null hypothesis, H_o = the treatment has no effect, versus an alternative hypothesis, H_A = the treatment has an effect. The P values of less than 0.05 were considered significant; however, due to small number of animals in some cases we discussed the data as being significant at $P < 0.1$. It is possible, that with a larger number of animals there would be a lower variance and some observed trends might have been significant at $P< 0.05$.

Figure X.1 Design of experiments 1, 2, 3, and 4.

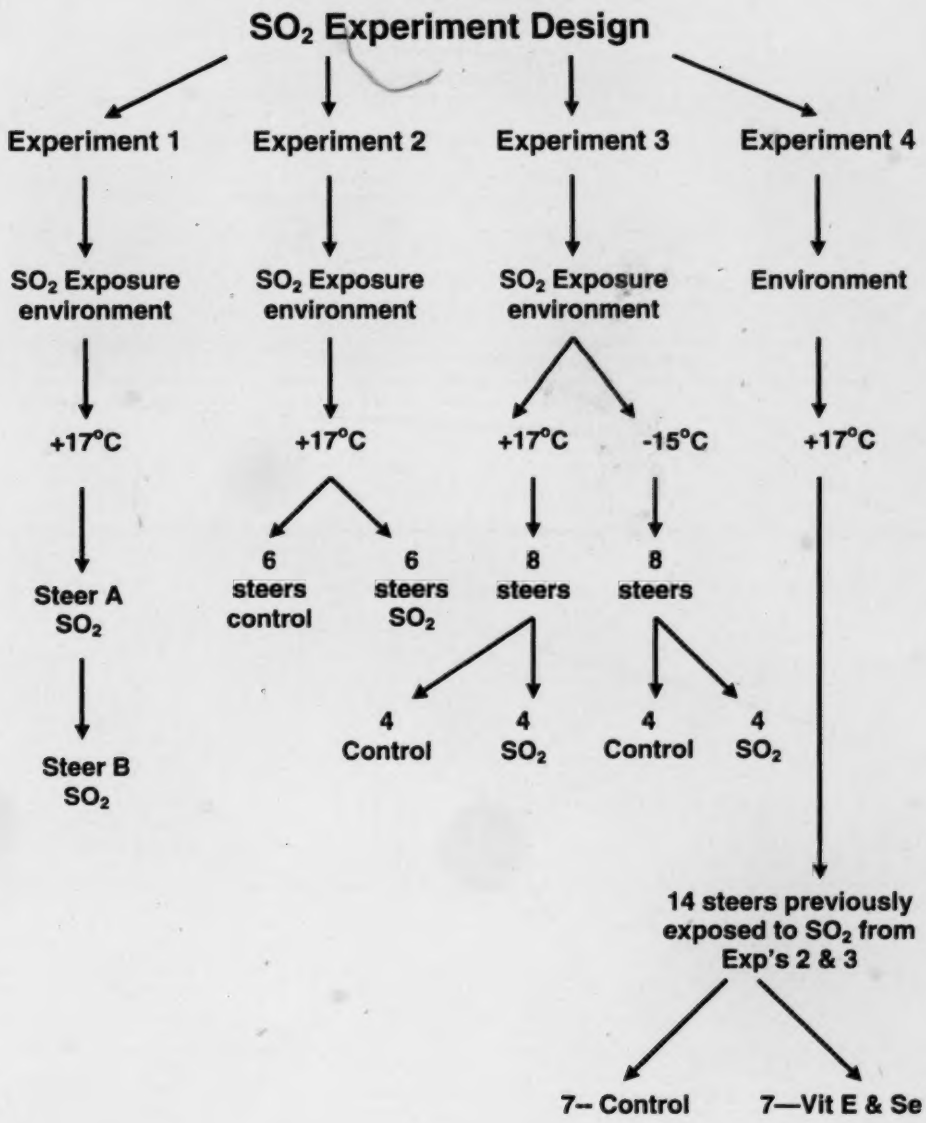


Figure X.2 SO_2 exposure periods, experiment 1

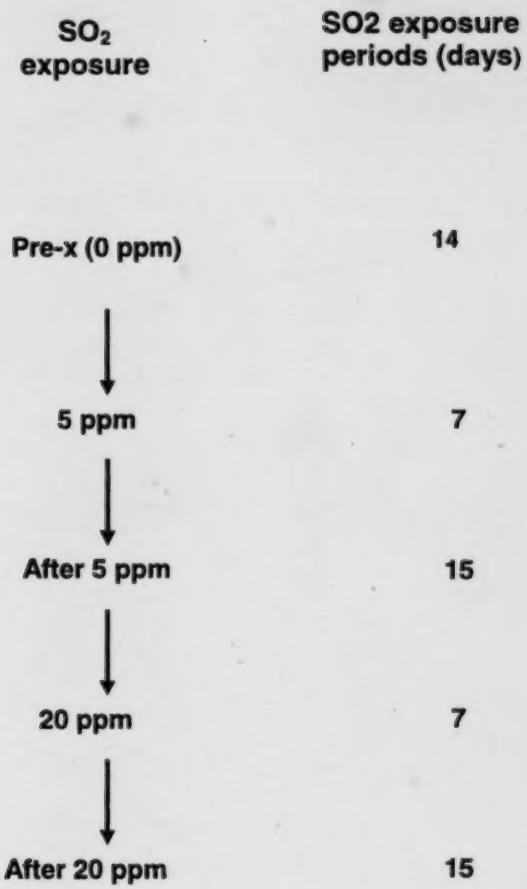
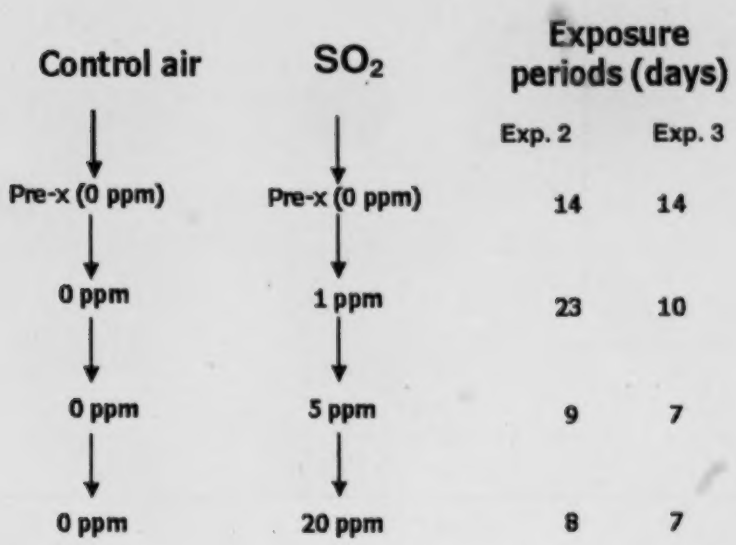


Figure X.3 SO_2 exposure periods, experiments 2 and 3



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Chapter 1

LITERATURE REVIEW

A. A REVIEW OF ISSUES RELATED TO SULFUR DIOXIDE INVOLVING ENVIRONMENTAL STRESS AND ENVIRONMENTAL POLLUTION

Air Pollution

The atmosphere is essential for survival of humans and all other species by providing carbon dioxide (CO_2) and nitrogen to plants and oxygen to animals. Ozone in the upper atmosphere helps protect the planet against excessive levels of ultraviolet radiation. Atmospheric precipitation provides moisture that enables plants to grow and animals to live. Our planet must have a clean atmosphere to be able to provide these essential components to sustain life.

Air pollution can occur as natural or as anthropogenic (resulting from the influence of human beings, man-made) event. Pollution disrupts the balance of gases in the atmosphere by increasing their amount, as in the case with CO_2, or may alter the composition of the atmosphere by adding new substances. Although there are many sources of anthropogenic air pollution, most of it is related to the combustion of fuels (Levetin and Van de 2001). In terms of the law of the conservation of matter, used fuel does not disappear; it is transformed into new substances, some of which are toxic over a wide range of concentrations. In addition, humans, animals and plants can be exposed to high concentrations of toxicants through industrial accidents involving the release of hazardous materials. A substantial number of toxic substances can also be released into the atmosphere by military action.

The debate about the safety of atmospheric pollutants is long-standing. The level of safety of some pollutants is unverified other than for laboratory animals. The major groups of primary air pollutants are listed in Table 1.1. Ambient pollution is usually a mixture of toxic compounds that combine together in the atmosphere through chemical reactions to form secondary pollutants (Langley-Evans *et al* 1996). As a result, it is difficult to discriminate between the collective and individual adverse effects of atmospheric pollutants on living organisms. For example, SO_2 absorbed onto particulates such as soot, forms a complex that is more toxic than SO_2 or particulate alone (Hippeli

and Elstner 1995). However, before testing a mixture, evidence must be presented regarding the toxicity of its distinct components. This thesis focuses on the immunologic and metabolic effects of one the major air pollutant, SO_2.

Sulfur Dioxide (SO2)

Sulfur dioxide is a colorless gas with the sharp odor of burning sulfur. Sulfur dioxide is about twice as heavy as air. Its chemical structure is O=S=O. Sulfur dioxide is released into atmosphere from industries that burn sulfur or heat metallic sulfur compounds, and from the burning of fossil fuels. Sulfur dioxide, a highly water-soluble gas, dissolves in water to form bisulfite (HSO_3^-), sulfite (SO_3) and hydrogen (H^+) ions. These reactions are described by the following equilibrium relationships:

$$SO_2 + H_2O \leftrightarrow H_2SO_4 \leftrightarrow HSO_3^- + H^+ \leftrightarrow SO_3 + 2H^+$$

The first suggestion that SO_2 may be a factor contributing to increases in mortality was raised in the middle of the 20th century in association with a pollution incident that occurred in Donora, Pennsylvania, in 1948 (Amdur 1974). Pollution from the Donora Zinc Works smelting operation and other sources containing sulfur, carbon monoxide and heavy metal dusts, was trapped by weather conditions in the narrow river valley in and around Donora and neighboring Webster. Thick smog created from a temperature inversion and the smoke laden with SO_2 from the factory blotted out Donora. Over several days, it killed 20 people and sickened 6,000 in one of America's greatest environmental disasters due to atmospheric pollution.

Province of Alberta, Canada

The province of Alberta is located in western Canada and is designated as Prairie. As a result of ancient sedimentation, compression and fermentation of organic matter in the soil, fossil fuel deposits were created throughout the province. As of 1994 a total of about 167,000 oil and gas wells had been drilled (Scott 1998). The raw gas processing capacity in the province in 1994 was over 1 billion m^3 of gas per day, including licensed processing capabilities of up to 32091 tonnes (t) of sulfur per day (Scott 1998). Over

65,000 gas and oil wells remained active as of 1996 (Scott 1998). The total industrial emissions of SO_2 in the province of Alberta, Canada in 1993 were 600,000 t per year (CASA 1997).

Maximum and threshold limit values (TLV) of SO_2

Threshold values are of prime importance in providing a sound basis for public health decisions. Humans and animals are capable of detoxifying most xenobiotics and restoring body functions that have been affected by internal or external exposures. However, the response of a living organism to a chemical exposure is limited. The Office of Air Quality Planning and Standards (OAQPS) have set standards for SO_2 gas which is one of the six "criteria" pollutants in ambient air. Units of measure for these standards are parts per million (ppm) by volume, milligrams per cubic meter of air (mg/m^3), and micrograms per cubic meter of air ($\mu g/m^3$).

In the Atmosphere

For Alberta, Canada, the ambient air quality guidelines for SO_2 were established by the Clean Air Act in 1984. These levels are 0.45 mg/m^3 or 450 $\mu g/m^3$ (0.172 ppm) per hour (as an 1-hour average concentration), 0.15 mg/m^3 or 150 $\mu g/m^3$ (0.057 ppm) per day (as a 24-hour average concentration), and 0.03 mg/m^3 or 30 $\mu g/m^3$ (0.011 ppm) per year (as an annual average concentration - arithmetic mean) (Alberta's Clean Air Act 1985).

In the workplace environment

In North America for humans, threshold limit values (TLV) for SO_2 in atmospheric air have been established to be 5.2 mg/m^3 (2 ppm) for a normal 8-hour workday or 40-hour workweek; workers may be repeatedly exposed to this amount without adverse effects (ACGIH 1996). 13 mg/m^3 (5 ppm) of SO_2 is a maximum concentration that should not be exceeded at any time during a 15-minute exposure period (ACGIH 1996).

Public and animal health

Humans

The validity of a risk assessment is based on the quality of the exposure assessment. Epidemiologic studies on air pollution use ambient pollutant concentrations as surrogates of personal exposure. Strong correlations among numerous ambient pollutant concentrations, however, have made it difficult to determine the relative contribution of each pollutant to a given health outcome (Sarnat *et al* 2001). During the summer of 1999, information was collected regarding respiratory health outcomes in 3,709 Chinese adults. Respiratory symptoms such as chronic cough, chronic phlegm, wheeze, and shortness of breath, but not physician-diagnosed asthma, were correlated with median indoor concentrations of SO_2 in three areas of China: Beijing: 14 $\mu g/m^3$ (0.006 ppm), Anqing City: 25 $\mu g/m^3$ (0.009 ppm), and rural Anqing: 20 $\mu g/m^3$ (0.008 ppm) (Venners *et al* 2001). Other studies have reported that 0.75 ppm of SO_2 in asthmatic subjects is a bronchoconstrictive stimulus (Wiebicke *et al* 1990). However, it has been recognized that there is a great amount of interindividual variation in response to SO_2 in asthmatic patients (Winterton *et al* 2001).

Laboratory animals

The literature describes the effects of SO_2 on several species of animals including mice, rats, guinea pigs, rabbits, dogs, cats, donkeys, and monkeys. An increase in airflow resistance and an alteration in mucus secretion are the two most significant effects that have been observed (Amdur 1974). Acute inhalation of SO_2 has been reported to increase rapidly airflow resistance in dogs, cats, and guinea pigs (Amdur 1974).

Ruminants

It has been reported that excessive quantities of dietary sulfur (above 0.3 to 0.4%), compared to recommended level of 0.15% in beef cattle diets (NRC 1996), supplemented as sulfate or elemental sulfur, may cause toxic effects and in extreme cases can be fatal in ruminants (Kandylis 1984). However, the harmful effect of SO_2 in ambient air on cattle, despite farmer's complaints, was not supported by the epidemiological studies conducted at Caroline, Alberta (Waldner 2001). Intensive biological accounting methods were used

to measure the health and productivity of cow-calf herds surrounding a new sour natural gas processing plant. The epidemiological examinations were performed on 7040 cow production records through a period from the fall of 1991 to the calving season of 1997 (Waldner 2001). No significant change in the risk of non-pregnancy, abortion, late calving, stillbirths, or calf mortality was reported after the gas plant became operational.

SO_2 biomarkers

In general, there are no obvious biomarkers for SO_2 exposure. However, it has been suggested that measurement of S-sulfonates in the nasal passage might serve as an effective biomarker for short-term exposure to SO_2 (Bechtold *et al* 1993). S-sulfonate level expressed as cyanolytically released sulfite was measured in the nasal lavage (NAL) fluid and blood plasma collected immediately after exposure of humans to 1 ppm of SO_2 for 10 minutes and to 7 ppm of SO_2 for 20 minutes. The levels of S-sulfonates observed in NAL fluid were almost three orders of magnitude higher than the S-sulfonates values measured in plasma (Bechtold *et al* 1993).

SO_2 sensitive subjects

Epidemiological studies have shown a relationship between air pollution and allergic airway disease. Sulfur dioxide is a common air pollutant and individuals with asthma are most sensitive to inhaled SO_2 (Riedel *et al* 1992). Considerable variation exists in the airway responses of asthmatics to the inhalation of SO_2 (Witek, Jr. and Schachter 1985), and bronchoconstriction seems to be the most common sensitivity response (Lester 1995). SO_2-induced bronchoconstriction is mediated by parasympathetic pathways (Sheppard *et al* 1980) and involves release of leukotrienes (Balmes *et al* 1987, Gong, Jr. *et al* 2001). An immunological mechanism of response to SO_2 also involves mast cells that line the surface mucosa of the lung. Axon reflexes are thought to be involved in bronchoconstriction induced by SO_2 in hyper-responsive asthmatic subjects (Rocchiccioli and Riley 1989). It was suggested that the wild-type allele of the tumor

necroses factor-alpha (TNF-α) promoter polymorphism may be associated with mechanisms of asthmatic sensitivity to inhaled SO_2 (Winterton *et al* 2001).

Human lungs develop throughout childhood until the age of 20 years old. Higher metabolic rates of children requires more oxygen exchange, which is compensated by a higher alveolar surface area to body mass ratio compared to adults resulting in a larger air-tissue gas exchange area. Thus, the lungs of children may be more susceptible to damage by air pollution (Boezen *et al* 1999). Table 1.2 summarizes the effect of SO_2 on the health of asthmatic subjects, children and elderly people.

Link to other diseases

Cancer

It was concluded from a variety of animal experiments that long-term exposure to SO_2 alone did not cause cancer (Melhlman 1983), and no clear evidence exists that SO_2 or bisulfite causes mutagenicity in mammals (Pool-Zobel *et al* 1990). On the other hand, it was found that workers exposed to SO_2 at a sulfuric acid factory in Taiyuan City (North China) had a higher number of lymphocytes with chromosomal aberrations (any abnormality of a chromosome's number or structure) compared to the subjects from another region that was free from SO_2 pollution. The observations that SO_2 is a possible clastogenic (causes breaks in chromosomes) and genotoxic (harms an organism by damaging its DNA) agent were supported by studies on mice bone marrow (Meng and Zhang 1990, Meng and Zhang 2002).

Chronic obstructive pulmonary disease (COPD)

Air pollution as a trigger for exacerbations of chronic obstructive pulmonary disease (COPD) has been recognized for more than 50 years, leading to the development of air quality standards in many countries that have, since, lead to substantial decreases in the levels of air pollutants such as black smoke and SO_2 derived from the burning of fossil fuels (MacNee and Donaldson 2000).

Diabetes

Subjects with diabetes mellitus may also be at risk for SO_2-induced toxicity according to studies performed on rats. Exposure of alloxan-induced diabetic rats (alloxan is a crystalline compound $C_4H_2N_2O_4$ causing diabetes mellitus when injected into experimental animals) to 10 ppm of SO_2 for 1 h/day, 7 d/wk, for 6 weeks increased lipid peroxidation, changed antioxidant enzyme activities and affected somatosensory evoked potentials components in diabetic rats by the increased release of free radicals compared to non-diabetic rats (Kucukatay *et al* 2003).

SO_2 in combination with air particulates

Air pollution is generally made up of a complex mixture of compounds, and various atmospheric conditions can alter the toxicity of air pollutants. In addition, the metabolism or detoxification of a single chemical in the body may be altered by the presence of other chemical pollutants as well as by the pre-existing health state of the affected organism itself (Oehme *et al* 1996). The toxicity of SO_2 in ambient air is significantly influenced by the coincident presence of particulates (Mehlman 1983). For example, protein-containing dust and SO_2 may promote toxic allergic reactions (Sosedova and Benemanskii 2000). The surface of inorganic particles contained in effluent gases produced during combustion of fossil fuels may interact with SO_2 to form an irritant aerosol. The interaction effect is dependent upon the size of particulates. The submicron fraction of this inorganic material may penetrate deeply into the lung and cause serious health effects (Peoples *et al* 1988). Table 1.3 summarizes the combined effect of SO_2 and other air pollutants on humans and other species.

The cold temperature and SO_2

It was found that even low levels of winter air pollution (50 $\mu g/m^3$ = 0.02 ppm of SO_2) encountered in Western Europe was associated with increased incidence of asthma attacks in children (Segala *et al* 1998). These observations are consistent with the studies conducted on subjects with mild asthma. It was suggested that breathing of dry cold air with 0.25 - 0.5 ppm of SO_2 significantly increased bronchoconstriction and specific

airway resistance (Bethel *et al* 1984, Sheppard *et al* 1984) compared to inhaling SO_2 or cold air alone. On the other hand, in guinea pigs, inhalation of clean cold dry air and cold air with 1 ppm of SO_2 produced similar cooling effects characterized by decreasing peak expiratory flow (Halinen *et al* 2000). Cold environments may influence immune function by suppressing lymphocytes expressing CD2, CD4 and CD4:CD8 cell ratio, and changing lymphocyte proliferative response to a T cell mitogen in sheep (Li *et al* 2000). However, the effect of prolonged and intense cold exposure on the immune system is not known. Mixture of black carbon aerosols and 10 ppm of SO_2 at relative humidity of 85% significantly suppressed alveolar macrophage phagocytosis in contrast to exposure at 10% relative humidity or to SO_2 alone at both levels of humidity (Jakab *et al* 1996).

The routes by which SO_2 enters living organisms

Air pollutants enter living organisms by inhalation through the nose and mouth and by contact with skin and mucous membranes (Oehme *et al* 1996). Humans and animals can be exposed to environmental pollutants through food (e.g. ingesting residues of pesticides used in agriculture), in the work place (e.g. inhalation of chemicals used in industrial processes), and directly from the environment (e.g. breathing polluted air). The respiratory system is the most common route for exposure of mammals to SO_2. On contact with moist mucous membranes, SO_2 forms sulfuric acid (H_2SO_4), which is responsible for its severe irritant effects on the eyes, mucous membranes, and skin (Amdur 1989). The rate of transfer from air to the mucosal lining depends on the ability of the gas to transfer in the liquid phase. The SO_2 solubility in water at 25°C and 1 atmospheric pressure is 0.4 g of SO_2 per 100 ml of water.

Organization of the airway epithelium

The respiratory tract extends from the external nares to the respiratory bronchioles (Frandson 1992). At the level of the larynx, it is divided into the upper and lower airways. The upper respiratory tract is connected to the paranasal sinuses, the eustachian tubes, and the mastoids (Frandson 1992). The lower tract is a series of conducting tubes

whose branching pattern varies from species to species. The upper and lower respiratory tracts are covered by a continuous layer of epithelium. Despite interspecies differences in the cellular composition of the respiratory tract epithelium, certain characteristics are shared by all species. Anterior nares of the upper airway are covered by squamous epithelium, while the epithelium of the lower airway is mostly ciliated, columnar, and pseudostratified. The airway lining layer, which is 95-98% water and rich in electrolytes, has several functions as a neutralizer for many toxins and an important component of the mucociliary apparatus. Its role as a diffusion barrier under normal conditions is questionable since this layer is only approximately 5 μm thick. However, in the hypersecretory state, such as in acute inflammation, this layer may increase several-fold in thickness (Hulbert *et al* 1982).

The effects of SO_2 on alveolar cells

The air sacs of the lung are lined by flattened epithelium (Reece 1997). Alveolar macrophages have also been identified as a distinct functional and morphological entity in the lung. The phagocytic activity of macrophages, in combination with the mucociliary clearance mechanism, helps to protect the lungs from intrinsic cell debris, inhaled dust, and microorganisms. Inhaled atmospheric pollutants decrease phagocytosis, leading to possible lung disease (Jakab *et al* 1996), (Ohtsuka *et al* 2000). Depending on the level and length of time of exposure, SO_2 and SO_3 may cause mucosal irritation, bronchoconstriction and coughing (Vai *et al* 1980).

The fate of SO_2 in human and animal organisms

Behavior and metabolism of SO_2

Sulfur dioxide is reactive with water and the hydrolysis of SO_2 occurs very rapidly in aqueous environments. Therefore, within fluid-filled structures such as cells, tissues, and blood vessels, any effects of SO_2 exposure must be due to the effects of bisulfite and/or sulfite anions. However, during inhalation of SO_2 gas, SO_2 itself will be present at the air-liquid interface, so the initial effects of SO_2 in cells at the luminal

surface of the airways could be due to the direct chemical effects of SO_2 gas rather than to the effects of bisulfite or sulfite (Amdur 1989). The two major hydrolysis products, sulfite and bisulfite, are present in roughly equal concentrations at physiologic pH as determined by the pKa of their equilibrium reaction (~7.2). However, at the pH of 6.6, reported at the luminal surface of the airways, the ratio of bisulfite to sulfite is approximately 5:1 and bisulfite is generally more chemically reactive than sulfite (Neta and Huie 1985). Bisulfite is a nucleophile that reacts with many biomolecules through substitution at electrophilic sites (Neta and Huie 1985). One of these reactions leads to the disruption of disulfide bonds and the production of thiosulfates (RSH) through the following reaction: $R\text{-}S\text{-}S\text{-}R + HSO_3^- \leftrightarrow RSSO_3^- + RSH$ (Petering and Shih 1975). Since disulfide bonds are widely found in tissue proteins, it is possible that bisulfite formed at the airway surface during SO_2 inhalation initiates bronchoconstriction by such an effect on surface proteins. In addition, sulfite ions ($SO_3^=$) in reaction with superoxides ($O^\bullet$) form very reactive sulfite radical ($SO_3^\bullet$) and hydrogen peroxide (H_2O_2). Sulfite radical ($SO_3^\bullet$) participates in radical chain processes such as lipid peroxidation (Hippeli and Elstner 1990). The chemical relationship between SO_2, $SO_3^\bullet$, and $SO_3^=$ has led to speculation that the bronchoconstriction that follows oral ingestion of sulfite-containing foods and beverages in some patients with asthma is mechanistically related to SO_2-induced bronchoconstriction (Stevenson and Simon 1984). The $SO_3^\bullet$ ion is converted to sulfate by sulfite oxidase, an enzyme found in the lung, the liver, and a variety of other tissues (Petering and Shih 1975). Sulfite can also be converted to sulfate by non-enzymatic autooxidation, which occurs in the presence of oxygen. This reaction is also a source of free radicals that may contribute to the tissue toxicity of SO_2 (Neta and Huie 1985).

Systemic effects and distribution

The distribution, metabolism, and toxicity of sulfite in the respiratory tract and other tissues have been studied on sulfite oxidase-deficient rats. The animals were exposed to 10 and 30 ppm of SO_2. The endogenously-generated sulfite and S-sulfonate compounds (a class of SO_2 metabolites) accumulated in the respiratory tract tissues and

in the plasma of these rats (Gunnison *et al* 1987). Additionally, their testes became severely atrophied and devoid of spermatogenic cells (Gunnison *et al* 1987). By contrast, compounds were restricted to the airways in normal, sulfite oxidase-competent rats exposed to the same concentrations of SO_2, sulfite and S-sulfonate (Gunnison *et al* 1987).

The clearance of SO_2 from a living organism

Mucociliary or pulmonary clearance

Mucociliary clearance of the respiratory tract is an important defense mechanism against inhaled pathogens and pollutants and depends on ciliary and mucous factors. Airway mucus, a complex airway secretion, functions as a renewable and transportable barrier against inhaled particulates and toxic agents. Inhalation of a number of air pollutants, including SO_2 and cigarette smoke, may increase mucus secretion and alter mucus rheology (Samet and Cheng 1994). Cilia, which line both the upper and lower airways, are covered by a thin layer of mucus and beat rapidly in a coordinated fashion propelling particles trapped in the mucus layer to the pharynx. Although no evidence for direct nervous control of ciliary function has been demonstrated, it was suggested that β-adrenergic agonists and digoxin might enhance ciliary function. Cilial defects may be either congenital (primary) or acquired (secondary) as a result of infection, toxins or drugs (Verdugo *et al* 1980). Degradation of the pulmonary surfactant dynamical interfacial properties due to inhalation of SO_2 polluted air may results in a slowdown of the pulmonary clearance rate and an increase in the lung burden of pollutant (Podgorski *et al* 2001).

Scrubbing

For highly soluble gases such as SO_2, the upper airways have been shown to be a very effective scrubber with much of the gas removed during a single pass. It was also noted that the concentration of SO_2 that induced bronchoconstriction in asthmatics did not affect lung function in normal subjects. It was observed in nine asthmatic teenagers subjected to 0.5 ppm of SO_2, that oral, and, to a lesser degree, oronasal inhalation elicited SO_2-induced changes in pulmonary function (Koenig *et al* 1983). Apparently, nasal

breathing, which is often impaired in asthmatics, reduces the pulmonary effects of SO_2 since this water-soluble gas is absorbed by the nasal mucosa (Peden 1997).

Enzymatic clearance by sulfite oxidase

Sulfite oxidase, a mitochondrial molybdo-enzyme in mammals, is essential for detoxication of the sulfite arising from metabolism of sulfur-containing amino acids, from ingestion of bisulfite preservatives and from inhalation of SO_2 (Coughlan 1983). Endogenous sulfite is generated as a consequence of the body's normal processing of sulfur-containing amino acids. Sulfites occur as a result of fermentation, and they also occur naturally in a number of foods and beverages. Despite the fact that they have been safely used as food additives for a long period of time, sulfiting food preservatives have been found to be allergy causative agents (Lester 1995). Sulfite sensitivity occurs most often in asthmatic adults and in preschool children (Lester 1995).

Uses of SO_2 in research to create models

Sulfur dioxide is extensively used on laboratory animals to create models for human airway diseases for the purpose of studying these pulmonary disorders. Table 1.4 summarizes uses of SO_2 in research to create models for human airway diseases

B. CLINICAL AND PHYSIOLOGIC RESPONSES TO SO_2

Clinical observations

Cardiovascular system

It has been reported that episodes of air pollution are associated with increases in hospital admissions for human cardiovascular disease. The Augsburg study (1984/1985) enrolled a cohort of 2681 men and women ranging from 25 to 64 years of age and monitored trends for determinants of cardiovascular disease. Heart rates were elevated at increased concentrations of SO_2 during the air pollution episode in January 1985

compared with non-episode days (Peters *et al* 1999). C-reactive protein concentration, an index of an acute phase response, was increased in 45-64 year old men in association with the 1985 air pollution episode and, in particular, with elevated concentrations of SO_2. These data indicate that one of the mechanisms for increased cardiovascular risk caused by air pollution is elevated acute phase responses (Peters *et al* 2001). One-hour exposure to 200 parts per billion (ppb) of SO_2 did not induce any changes in human lung function. However alterations in the electrocardiogram were evident (Tunnicliffe *et al* 2001).

Dietary protein effects and feed intake

Glutathione (GSH) plays a major role in the detoxification of SO_2 within the lungs. The maternal diet is an important determinant of glutathione-related metabolism in rats. The effects of fetal exposure to a low protein maternal diet upon later susceptibility to pulmonary injury induced by chronic SO_2 exposure were evaluated in young adult rats (Langley-Evans *et al* 1997). Pregnant rats were fed purified diets containing graded amounts of protein. At 7 weeks of age, male pups were housed in room air or 286 $\mu g /m^3$ (0.1 ppm) of SO_2 for 5 hours per day during a 28-day period. The rats exposed to low protein diets *in utero* exhibited significantly greater pulmonary injury. Differences in susceptibility to SO_2-induced tissue injury may be related to programming of GSH metabolism by the maternal diet (Langley-Evans *et al* 1997). Other studies have reported mice to exhibit a reduced activity and a slight but significant decrease in water and food consumption upon exposure to air polluted with SO_2 emitted from a coal-fired power plant (Gorriz *et al* 1996).

Body weight

It was found that the low birth weight of term babies born between 1 January 1994 and 31 December 1996 in six northeastern cities of the United States: Boston and Springfield, Massachusetts; Hartford, Connecticut; Philadelphia and Pittsburgh, Pennsylvania; and Washington, DC was associated with increased levels of SO_2 in ambient air (Maisonet *et al* 2001). However, this effect could not be shown in SO_2 exposure experiments with rats (Klein *et al* 1989).

Respiratory function

Various lung measurements have been used to assess the response to inhaled SO_2 in controlled laboratory studies. The volume of air exhaled in the first second of a forced expiratory maneuver (FEV_1) and specific airway resistance (SRaw) are two of the most widely used tests of lung function. Sulfur dioxide, ozone, sulfuric acid, and nitrogen dioxide exposures are associated with reduced FEV_1 (Schwela 2000). Sulfur dioxide is also a precursor of secondary sulfates such as sulfuric acid, which is a stronger irritant than SO_2, and plays a major role in the adverse respiratory effects of air pollution (Donoghue and Thomas 1999). Sulfate is a major component of $PM_{2.5}$ (particulate matter of 2.5 micron in diameter) which has been implicated in causing adverse health effects, especially among the elderly and persons with cardiovascular and respiratory illnesses. Asthmatic subjects exposed to levels of SO_2 within regulatory standards have demonstrated increased respiratory symptoms such as shortness of breath, coughing and wheezing, and decrements in lung function relative to controls (Donoghue and Thomas 1999).

Energy Metabolism

A living organism is an open system, which continuously interacts with the environment and, therefore, conforms to the laws of thermodynamics in terms of conservation of energy and spontaneous increase in randomness or entropy. Tissue cells and molecules undergo spontaneous turnover with some being destroyed because of entropy. As a result, living organisms require a continuous supply of energy in order to maintain homeostasis (Hochachka and McClelland 1997). This energy plays a major part in enabling farm animals to produce the desired products. A considerable part of the total feed is needed for maintenance of ingestion, absorption, regulation, detoxification, and utilization of the nutrients. Severe injuries can result in a 15 to 30% loss of body weight, but the protein contribution to caloric expenditure does not exceed 20% and is less than expected (Long 1977). The provision of calories and nitrogen can change the course of the septic patient (Long 1977). Fever and infection also result in increased metabolic heat production (Baracos *et al* 1987). This response contributes, with reduced dietary energy

intake, to negative energy balance in the infected host and constitutes a metabolic "cost" (Baracos et al 1987). Heat is a very important byproduct of energy metabolism; it permits homeotherms to maintain a body temperature much above the temperature of the environment. Utilization of feed energy involves energy losses in feces, urine, combustible gases and heat. Maintenance metabolism is a large part of total energy metabolism exceeding 60% in ruminating growing cattle (NRC 1996). The environmental temperature may affect body metabolism resulting in alteration of some physiological parameters. Thus, body weight gains, rectal temperatures, respiratory rates and water intakes were significantly lower in young calves housed at colder temperatures (-4°C) compared to animals housed at 10°C. Calves housed at -4°C had also higher maintenance energy requirements (0.133 Mcal metabolizable energy/kg^{75}) than calves housed at 10°C (0.101 Mcal metabolizable energy/$kg^{0.75}$) (Scibilia *et al* 1987). In young calves, the increase in heat loss upon standing in the cold was greater than the increase in heat production upon standing in a thermo-neutral environment. As a result, young calves require increased cold-induced thermogenesis when they are standing (Schrama 1983). Studies performed with beef bulls in Western Canada support the statement that cold stress, poor or excessive body condition, and reduced feed quality interact with normal physiological processes, which may result in reduced semen quality in the winter months (Barth 2002). It appears that when feed intake is limited in cold-adapted animals, muscle and skin have a lower priority for nutrients than other organs and tissues, resulting in reduced protein synthesis (Scott *et al* 1993). While the effects of cold stress are known, the effect of chemical stress on energy metabolism in humans and animals is still largely uncharacterized. There is a need to quantify effects of air pollutants on energy metabolism in livestock, since there is potential to alter feed requirements.

Free radicals

Living organisms are dependent on oxygen (O_2) as a catalyst to release energy from substrate for many cellular functions including maintenance, metabolism, and repair. In addition, O_2 participates in many oxidative reactions involving the cytochrome P-450 system, hydroxylases, and oxidases (Kovalev *et al* 1990). These enzymes are very important in the metabolism of drugs, xenobiotics, and other metabolic intermediates. Polymorphonuclear cells (PMNs), which are the first lines of defense against infection, require O_2 to kill microorganisms by producing free radicals such as superoxide ($O^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen, and other products via the respiratory burst (Babior 1978). The PMN cell has an inherent protective mechanism to detoxify these free radicals since they possess endogenous oxidants including superoxide dismutase (SOD), catalase, and glutathione (GSH). It has been shown that the degree of PMN cell function in killing of bacteria is directly dependent on O_2 tensions (Dechatelet 1975). PMN cells also have O_2 independent killing pathways through lysozymes, acidic vacuoles, and lactoferrins. However, these pathways are less efficient and vary significantly depending on the organism.

Biochemical and Physiological Parameters

Several epidemiological and clinical controlled studies have investigated effects of SO_2 on the red blood cells (RBC) and white blood cells (WBC) count and the level of proteins and vitamins and enzyme activity in blood serum of humans and various animal species. Table 1.5 summarizes effects of SO_2 on a number of biochemical and hematological parameters in humans, rats, guinea pigs, mice, birds, and monkeys. The tested concentrations of SO_2 were from 0.1 ppm to 400 ppm.

C. IMMUNE SYSTEM

The anatomy of the immune system

Historically, immunity has been divided into two major categories, innate (natural) immunity and acquired (adaptive) immunity (Kuby 1997). Innate immunity is that which develops early in life and does not maintain a memory in response to exposure. Its elements include physical defenses such as the skin, mucous membranes and the cough reflex, and chemical elements, the examples of which are low pH, digestive enzymes and secreted fatty acids. If the physical barriers are breached, cellular components of the innate immune system respond primarily through phagocytosis of bacteria by macrophages, or cytolysis of virus-infected host cells by natural killer (NK) cells. In contrast to innate immunity, acquired immunity is specific and has memory; therefore, upon secondary contact with an infectious agent, the immune response will occur faster and will be stronger than upon first exposure to the same agent (Sweat 1983). The acquired immune response is mediated by white blood cells (leukocytes). They include B lymphocytes and activated B cells called plasma cells which produce antigen-specific antibodies, as well as T lymphocyte subpopulations that secrete cytokines and are cytotoxic against some virally infected cells, tumors and foreign tissue grafts (Minatel and Carfagnini 2000).

The immune system operates throughout the body. There are, however, certain sites where the cells of the immune system are organized into specific structures. These are classified as central lymphoid tissue (bone marrow, thymus) and peripheral lymphoid tissue (lymph nodes, spleen, mucosa-associated lymphoid tissue). A variety of substances inhaled in air and ingested as foods can trigger immune responses. One of the functions of immune system is to protect against foreign substances by utilizing different defense strategies. Immune defense is a continuous process associated with a benefit and a cost. The negative side of immunity is characterized by the fact that increased production of free radicals designed to kill invading pathogens may also damage healthy host tissues leading to a variety of illnesses (Hippeli and Elstner 1995).

Immune activation

Activation of immune cells can be specific, by antigens, which alert only cells armed with a specific receptor; or, activation can be non-adaptive with whole populations of cells turned-on by cell-stimulating mediators, such as interleukin 2 (Dong and Flavell 2000). The non-specific stimulus might be a bacterial or viral infection, a drug-induced allergic event, parturition, surgery, or exposure to an environmental toxin. It is suggested that hypersensitivity is enhanced by increased toxicity of the environment (Dong and Flavell 2000). Environmental cycles such as light (Goldman 2001) and temperature (Shephard and Shek 1998) influence immune responses. Certain immune cells act as sensors, which trigger emergency responses in the presence of antigens. Other cells are effectors that are specialized in recruiting and activating other populations of cells, which carry out defensive actions. Some cells are both sensors and effectors. Other cells are modulators that regulate the reactivity of sensor and effector cells (Dong and Flavell 2000).

Immune cells communicate with each other via a set of chemical signals (cytokines) that are received by receptors on cell surfaces (Bilbo et al 2002). Immune cell populations are large and dispersed; therefore, cytokines are of a very general nature and may reach millions to billions of cells during any given event. Some signals cause cell populations to migrate; other messages trigger rapid proliferation of cells and/or change the appearance and behavior of cells during an activation sequence (Bilbo et al 2002).

Lymphoid lineage of immune cells

Lymphocytes range in size from 6-10 μm in diameter and subtypes are relatively indistinguishable by light microscopy. The cells express a large number of different protein "markers" on their surfaces that can be used to distinguish among the subpopulations. The presence of the markers can be detected using specific labeled monoclonal antibodies (Mab) that bind to them. A systematic nomenclature (the "cluster of differentiation/designation" or "CD" system) has been developed to identify the markers (Goldstein 1982). It is possible to accurately distinguish lymphocytes from other leukocyte populations in peripheral blood with flow cytometry technique using forward

and orthogonal light scatters mounted inside a flow cytometer. In order to identify the cell population of interest based on immunofluorescence, a gate (a light scattering window) is set to include the majority of the lymphocytes and another gate is selected for the monocytes. In this manner, maximal recovery of the lymphocytes and non-lymphocytes within a sample can be consistently obtained (Loken et al 1990). The immuno-phenotyping of lymphocyte subsets may be influenced by a variety of technical factors, which include the duration and temperature of sample storage and the method used for staining samples (Ekong et al 1993).

T cells

T lymphocytes arise in the bone marrow, then migrate to and mature in the thymus (Felsburg 2002). All T cells possess the T cell antigen receptor (TcR), which serves as the T cell's definitive marker (Call 2002). In addition, all T cells possess the CD3 marker, a complex of 5 polypeptides (Felsburg 2002). There are two major types of T cells, helper and cytotoxic T cells. Helper T cells (Th) secrete cytokines that promote the proliferation and differentiation of T cells, B cells and macrophages, and that recruit and activate inflammatory leukocytes (Felsburg 2002). Helper T cells are identified by the presence of the CD4 marker (Felsburg 2002). Cytotoxic or cytolytic T cells (Tc) lyse cells that produce foreign antigens, e.g. tumor cells, virus-infected cells, and foreign tissue grafts, thus, initiating cell-mediated immunity. Cytotoxic T cells are identified by the presence of the CD8 marker (Felsburg 2002).

There are also two forms of TcR - α/β TcR and γ/δ TcR (the Greek letters refer to the polypeptide chains from which the TcR are formed). T cell recognition of antigen involves direct cell-cell contact between the antigen-specific TcR on the T lymphocyte and an MHC/peptide complex at the surface of a MHC compatible antigen presenting cell (MHC is a major histocompatability complex). In general, class I MHC molecules present antigen to $CD8^+$ T cells, and class II MHC molecules present antigen to $CD4^+$ T cells. α/β^+ TcR cells express either the CD4 or the CD8 surface marker. Class I MHC molecules are expressed on almost all nucleated cells of the body, while expression of class II molecules is restricted to B cells, macrophages, dendritic and certain other cells

of the immune system. It is not clear how γ/δ^+ T cells recognize antigen, however most γ/δ^+ T cells do not appear to be restricted by MHC molecules (Sopp and Howard 2001).

B Cells

B lymphocytes acquired their name because in birds they were first shown to mature in the Bursa of Fabricius (Davison 2003). In mammals, the early stages of B cell maturation occur in the fetal liver and bone marrow. Surface immunoglobulin (sIg) is the B cell's receptor for antigen and its definitive marker (Minatel and Carfagnini 2000). Additional markers that distinguish B cells include CD19, CD20, CD21 and CD22. B cells may differentiate into plasma cells, which secrete large amounts of antibodies, or into memory B cells (Minatel and Carfagnini 2000). Upon secondary contact with antigen, memory B cells proliferate rapidly and begin active antibody secretion. T cells can also form memory cells (Minatel and Carfagnini 2000).

Bovine Mab's

Extensive studies of cells of the immune system have employed Mab's to phenotype cells and to define their functions. It has become evident that lymphocyte subpopulations in different animal species are phenotypically and functionally homologous (Davis at al 1993). Workshop cluster (WC) is the nomenclature system for Mab's/antigens where homology with human cluster of differentiation (CD) antigens has not been established. In cattle the major subpopulations of T lymphocytes identified in peripheral blood are $BoT2^+$, $BoT4^+$, $BoT8^-$ (20-40% of blood lymphocytes) and $BoT2^+$, $BoT4^-$, $BoT8^+$ (10-32% of blood lymphocytes) that are homologues of the $CD2^+$, $CD4^+$, $CD8^-$ and $CD2^+$, $CD4^-$, $CD8^+$ cells respectively. Table I.6 illustrates monoclonal antibodies (Mab's) as markers for identification of bovine peripheral blood leukocyte subpopulations (Davis et al 1993).

Myeloid lineage of immune cells

Generally, there are two major types of myeloid cells, the mononuclear phagocytes (monocytes and macrophages) and the granulocytes (neutrophils, eosinophils, basophils and mast cells) that mediate inflammation by releasing their granule contents. Phagocytes (monocytes, neutrophils, and eosinophils) are the cells that engulf and destroy foreign substances. Differentiation of the myeloid progenitor into each committed cell type depends upon which interleukins (ILs) and colony stimulating factors (CSF) are present in the milieu where the cells are maturating. Sources of the ILs and CSFs include stromal cells and mature myeloid and lymphoid cells (Minatel and Carfagnini 2000).

Mononuclear phagocytes (monocytes)

Mononuclear phagocytes (monocytes) have horseshoe-shaped nuclei and circulate freely in the blood. When a monocyte passes out of the blood vessels and becomes fixed in a tissue, it is matures to a macrophage. Tissue-fixed phagocytic cells form a multi-organ network called the mononuclear phagocyte system, formerly known as the reticuloendothelial system. Mononuclear phagocyte system include Kupffer cells in the liver, alveolar macrophages in the lung, splenic and peritoneal macrophages, microglial cells in the brain, osteoclasts in the bones, dendritic cells in the lymphoid organs, Langerhans cells in the skin and mesangial cells in the kidneys (Sopp et al 1996).

Polymorphonuclear granulocytes (neutrophils)

Neutrophils are characterized by their multilobed nuclei and azurophilic granules. They develop from the finely granular myelocytes of the bone marrow. Their primary function is phagocytosis and an important secondary function is to activate macrophages at a wound/infection site. Neutrophils are the most actively migrating cells in acute inflammatory responses. They migrate through the endothelial cell junctions into the inflammatory exudates in large numbers and are most concerned with pyogenic infections associated with bacteria. Like the mononuclear cells, neutrophils produce reactive oxygen metabolites and hydrolytic enzymes (Rainard *et al* 2000). In addition,

neutrophils produce nitric oxide and antibiotic proteins such as defensins, seprocidins, cathelicidins, and bacterial permeability inducing protein. It was suggested that protein kinase C, beta-adrenergic receptors, G-proteins, protein tyrosine kinase(s) and Ca^{2+} uptake, may all be involved in some part of the process of bovine neutrophil activation (Yu and Czuprynski 1996)

An influx of neutrophils into the airways is a common feature observed during pulmonary inflammation induced by air pollutants, including SO_2 and sulfates (Labbe *et al* 1998). Based on an *in vitro* study sulfite at low concentrations (0.01-1 mM) was able to activate the respiratory burst of neutrophils to produce superoxide anions (O^{2-}) and H_2O_2. However, in higher concentrations (2-10 mM) the production of free radicals was reduced. Sulfate and hydrated forms of SO_2, had no effect on respiratory activity of blood neutrophils (Beck-Speier *et al* 1994).

Soluble mediators of immunity (humoral immunity)

The definition of humoral immunity refers to any extracellular, soluble factors such as acute phase proteins, cytokines, and antibodies found in the blood or lymph that contribute to host protection (Ahmed 2001).

Acute phase response

The acute phase response is stimulated by the release of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) from macrophages and monocytes at the site of inflammatory lesions or infection (Mackiewicz *et al* 1992). These cytokines travel through the blood and stimulate hepatocytes in the liver to synthesize and secrete acute phase proteins. This response provides an early defense by enabling the body to recognize foreign substances early on in the infection process (Mackiewicz *et al* 1992).

Acute phase proteins (APP)

Acute phase proteins (APP's) are a large and varied group of glycoproteins in the serum, which are unrelated to immunoglobulins. These are a group of plasma proteins,

many of which are produced by the liver (Moshage 1997). Generally, concentrations of APP's increase several-fold during an inflammatory response. They are found in all serum protein fractions except albumin. C-reactive protein (CRP), haptoglobin (Hpt), and α-1 acid glycoprotein (AGP or orosomucoid) are valuable biomarkers of inflammation and infection. AGP is classified as a moderate responder in the dog, pig and cow and a major responder in the rat (Jensen, Whitehead, 1998). In bovine medicine, the principle acute phase proteins to be used are Hpt and AGP. Haptoglobin was elevated in several important bovine infectious diseases such as pasteurellosis (Horadagoda *et al* 1994), pneumonia (Wittum *et al* 1996), mastitis (Hirvonen *et al* 1996), foot and mouth disease virus (Hofner *et al* 1994) and fatty liver (Nakagawa *et al* 1997). In 81 cattle, Hpt was elevated in acute and AGP in chronic inflammation (Horadagoda *et al* 1999). The assay of determination of Hpt in bovine serum or plasma is based on the binding of Hpt to hemoglobin (Hb) (Godson *et al* 1996).

D. CORTISOL

Cortisol is a steroid hormone derived from cholesterol, which is synthesized in the adrenal gland (see Figure 1.1 for a chemical structure of the hormone cortisol). Its level in the blood is controlled by negative feedback systems in the hypothalamus and pituitary gland. Cortisol, released in the body during stressed or agitated states, has gained widespread attention as a "stress hormone." However, this hormone is not just a simple marker of stress levels-- it is necessary for the functioning of almost every part of the body. Excesses or deficiencies of this crucial hormone also lead to various physical symptoms and disease states. Among its important functions in the body include roles in the regulation of blood pressure and cardiovascular function as well as regulation of the body's use of proteins, carbohydrates, and fats (Johnson and Vanjonack 1976). Cortisol secretion increases in response to any stress whether physical (such as illness, trauma, surgery, or temperature extremes) or psychological (Uchino et al 2001). The influence of acute exposure to thermal stress on the secretion of cortisol was monitored in 14 lactating Holstein cows (Wise *et al* 1988). Cows were maintained throughout the summer in a refrigerated air-conditioned tie stall barn or in outdoor corrals with access only to shade.

Serum cortisol concentrations were higher in heat-stressed cows compared to cows maintained under cooling (Wise *et al* 1988). Cold stress also increased plasma cortisol levels for less than 1 hour in six healthy neonatal calves chilled with cold water (Slocombe *et al* 1984) and increased cortisol metabolic clearance rate in sheep (Graham et al 1981). Surgical stress in 20 dairy cows led to extremely increased plasma cortisol levels within 5 hours after the surgery (Mudron *et al* 1994). A literature search did not reveal any information on the effect of air pollutants on concentration of cortisol in blood humans and animals.

E. SELENIUM

The early interest in selenium (Se) was related primarily to its toxicity, but since 1957 the element has been recognized as a dietary essential (Ammerman and Miller 1975). The dietary requirement for Se by cattle is 0.3 – 1.0 ppm dry matter (Puls 1994) that is most beneficial for performance of immune and reproductive systems. With adequate dietary intake serum will contain about 30% of whole blood Se (Puls 1994). Deficiencies of Se in cattle and sheep have been confirmed under natural grazing conditions in many countries of the world. Obvious signs of insufficiency such as white muscle disease (nutritional muscular dystrophy) occur primarily in young calves or lambs born to Se deficient dams (Puls 1994). Infertility may increase in dams grazing pastures low in Se. Concentration of Se in tissue, particularly in the liver, has been used in establishing Se status of the cattle (Puls 1994). The dietary requirements for Se and its metabolism are influenced by many nutrient interrelationships, including its interactions with sulfur, lipids, vitamin E, proteins, amino acids, and several microelements (Ammerman and Miller 1975).

Selenium deficiency is responsible for Zenker type muscle degeneration in calves, lambs, and foals in the prenatal and postnatal stages of development (Bostedt and Schramel 1990). Postnatal nutritive muscular dystrophy, however, is attributed to both acute Se and vitamin E deficiency in basic feed or impaired plant absorption of Se as a result of antagonistic elements, such as sulfur (Bostedt and Schramel 1990). The authors investigated the effect of altered Se nutrition on arachidonic acid oxidation in immune

cells. Experiments were conducted with blood cells obtained from dairy cattle fed either diets supplemented with or deficient in Se. The results indicate that the concanavalin A-stimulated lymphocyte proliferation was significantly lower in Se-deficient cows. When stimulated by calcium ionophore A23187, the lymphocytes derived from Se-deficient cows produced significantly less 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B4 (LTB4) than those obtained from Se-supplemented cows. When included in cell cultures from animals fed +Se diets, 5-HETE and LTB4 elicited a partial reversal of the inhibition of lymphocyte proliferation by either hydrocortisone or nordihydroguaiaretic acid. Studies on cattle concluded that dietary Se status, which in turn determines tissue Se concentration, plays an important role in the regulation of arachidonate metabolism in peripheral blood lymphocytes. This may be one of the biochemical mechanisms underlying the inhibition of lymphocyte proliferation and the decrease in resistance to infectious diseases observed in Se-deficient animals (Cao *et al* 1992).

F. VITAMIN E

Vitamin E is a mixture of eight related compounds known as tocopherols that are synthesized by plants. The α-tocopherol molecule (see Figure 1.2 for a chemical structure of α-tocopherol) is the most potent of the tocopherols. Vitamin E is absorbed from the intestines packaged in chylomicrons. It is delivered to the tissues via chylomicron transport and then to the liver through chylomicron remnant uptake. Due to its lipophilic nature, vitamin E accumulates in cellular membranes, fat deposits and other circulating lipoproteins. The major site of vitamin E storage is in adipose tissue. Vitamin E acts as a natural antioxidant by scavenging free radicals and molecular oxygen. In particular vitamin E is important for preventing peroxidation of membrane polyunsaturated fatty acids in neurons (Tamagno *et al* 2000). The vitamins E and C are interrelated in their antioxidant capabilities. Active α-tocopherol can be regenerated by interaction with vitamin C following scavenge of a peroxy free radical (Lee and Dabrowski 2003). Alternatively, α-tocopherol can scavenge two peroxy free radicals and then be conjugated to glucuronate for excretion in the bile (Basu and Dickerson 1996).

Pollution is a stressor that may deleteriously affect immune function of the body. Thus, inhalation at 10 ppm SO_2 for 1 h/day, for 30 days induced oxidant stress in red blood cells of guinea pigs (Etlik *et al* 1995). The administration, once in every 3 days, of vitamin E (40 mg/kg) together with vitamin C (200 mg/kg) reduced the damage in erythrocyte membranes (Etlik *et al* 1995). Seventeen adult asthmatic subjects with ozone-induced bronchial hyperresponsiveness were subjected to 0.10 ppm and 0.25 ppm SO_2 for 10 min. The patients who were given dietary antioxidants (400 IU vitamin E and 500 mg vitamin C) responded less severely to SO_2 challenge than control patients who were given a placebo. The results suggest that dietary supplementation with vitamins E and C benefits asthmatic adults who are exposed to air pollutants, and in particular to SO_2 (Trenga *et al* 2001). The role of dietary interventions such as vitamin E and selenium against tissue injury by oxidizing gases has been addressed by several investigators (Goldstein *et al* 1970), (Menzel *et al* 1972), (Elsayed *et al* 1983), (McMillan and Boyd 1982). It has been postulated that the antioxidant properties may be the result of vitamin E being preferentially oxidized by the oxidizing gas, thereby preventing the formation of lipid peroxides, or vitamin E may react with free radicals produced by lipid peroxidation (Dillard *et al* 1982).

G. VITAMIN A

Vitamin A (or Retinol) consists of three biologically active molecules, retinol, retinal (retinaldehyde) and retinoic acid (Wingerath et al 1999). Figure 3 illustrate a chemical structure of vitamin A. Each of these compounds are derived from the plant precursor molecule, β-carotene. Ingested β-carotene is cleaved in the lumen of the intestine by β-carotene dioxygenase to yield retinal. Retinal is reduced to retinol by retinaldehyde reductase, an NADPH requiring enzyme within the intestines (Siegenthaler *et al* 1990). Retinol is esterified to palmitic acid and delivered to the blood via chylomicrons. The uptake of chylomicron remnants by the liver results in delivery of retinol to this organ for storage as a lipid ester within hepatocytes. Transport of retinol from the liver to extrahepatic tissues occurs by binding of hydrolyzed retinol to aporetinol binding protein (RBP). The retinol-RBP complex is then transported to the cell

surface within the Golgi and secreted. Within extrahepatic tissues retinol is bound to cellular retinol binding protein. Plasma transport of retinoic acid is accomplished by binding to albumin (Basu and Dickerson 1996).

Retinol is an essential nutrient which has many physiological effects throughout the body. It is stored in the liver and deficiency of the vitamin occurs only after prolonged lack of dietary intake. Vitamin A is necessary for support of growth, reproduction, and maintenance of higher animals. In the absence of vitamin A, animals will stop to grow and eventually die (Frye et al 1991). More is known about the role of vitamin A in vision than any of its other functions. However, it was suggested that beta-carotene plays a role in bovine health and reproduction (Chew et al 1983). *In vitro* assays that emphasize cellular components critical to the host defense system indicate that vitamin A is required for epithelial cell differentiation (Fisher and Placker 1987). It was also shown in rats that retinol may decrease the inflammatory response through inhibition of pulmonary macrophage function, thus resulting in decreased chemically-induced pulmonary injury (Sauer et al 1995).

H. SUMMARY

Sulfur dioxide is a ubiquitous air pollutant encountered in significant concentrations in outdoor and indoor air and in industry. The most important health effect of SO_2 is that it causes bronchoconstriction in people with asthma, especially when inhaled during exercise. In aqueous environments, SO_2 is rapidly hydrolyzed to form sulfite, bisulfite, and hydrogen ions. Sulfite and hydrogen ions are not likely to cause SO_2-induced bronchoconstriction, but the relative importance of bisulfite and SO_2 gas itself remains to be determined, as does the initial cellular site of action of either of these forms of SO_2. The focus of public health concern and research in regard to environmental lung diseases has changed across the century. It has shifted from avoiding clinical disease among highly exposed individuals to protecting the population from an unacceptable burden of risk (Samet and Utell 1991). Epidemiological studies suggest that SO_2 pollution has subtle effect on health of cattle.

Figure 1.1 Chemical structure of cortisol

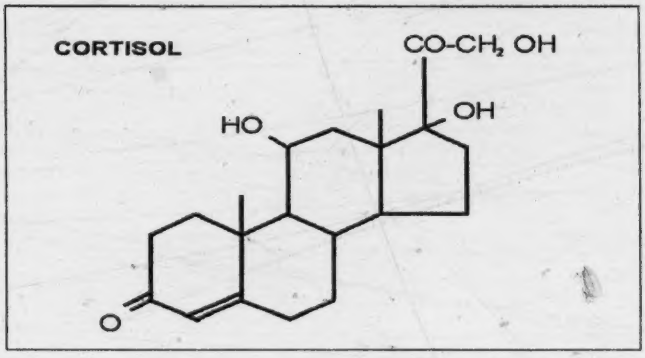

Figure 1.2 Chemical structure of α-tocopherol

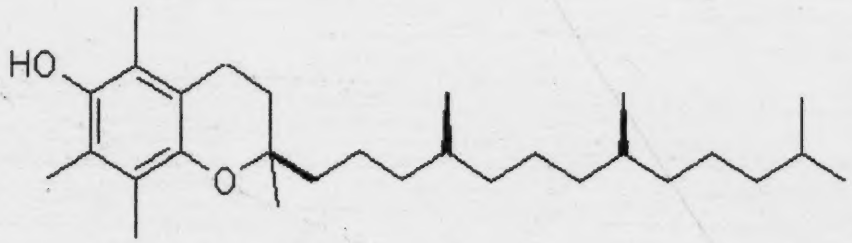

Figure 1.3 Chemical structure of the vitamin A (retinol)

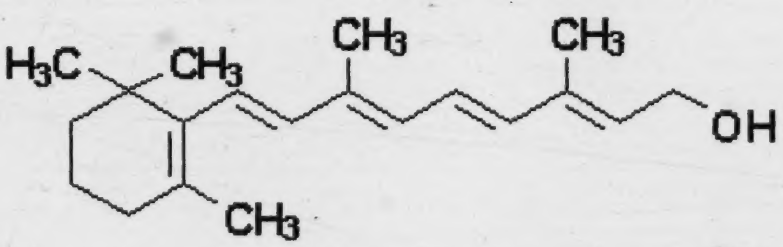

Table 1.1 The major groups of primary air pollutants

Name of the air pollutant	Common chemical formula	Main characteristics	Main sources
Carbon dioxide	CO_2	Greenhouse gas	Combustion of fossil fuels and burning of vegetation.
Carbon monoxide	CO	Toxic odorless gas, product of the incomplete combustion of carbon	Automobiles, planes, furnaces
Sulfur oxides	SO_x	Component of acid rain	Burning of fossil fuels
Nitrogen oxides	NO_x	Component of acid rain	Automobiles, 0poer stations, furnaces
Hydrocarbons	Alkanes (C_nH_{2n+2}.); Alkenes (C_nH_{2n}.); Alkynes (C_nH_{2n-2})	Gaseous forms	Petrochemical industry, Combustion of fossil fuels
Particulates	Solid or liquid matter less than 0.1 mm in diameter that is suspended in the air	Coarse particles are larger than 0.002 mm; fine particles are smaller than 0.002 mm	Coarse particles--road dust, sea spray, and construction; fine particles--automobiles, coal-fired power plants, oil refining, wood stoves

Table 1.2 Effect of SO_2 on the health of asthmatic subjects, children and elderly people

Species, Subjects	Research	SO_2 concentration	Units	Duration	Effect	Reference
Mild asthma	trial	0.25, 0.5, 1.0	ppm	10 min	bronchoconstriction	Sheppard et al (1980)
asthmatics	trial	0.5	ppm	10 min	bronchoconstriction	Winterton *et al* (2001)
Non-allergic asthma	trial	0.5, 1.0	ppm	20 min	bronchoconstriction	McManus *et al* (1989)
Asthmatic	trial	2.0	ppm	30 min	bronchoconstriction	Bedi and Horvath (1989)
Healthy non-smokers	trial	2.0	ppm	30 min	no	Bedi and Horvath 1989)
asthmatics	trial	1.0	ppm	2 h	Lung ventilatory function ↓	Mehlman (1983)
children	epidem	pollution		10 years	bronchitis	Herbarth *et al* (2001)
adolescents	trial	1.0	ppm		bronchospasm	Koenig *et al* (1982)
Asthmatic children	trial	70 - 120	ppb		no	Huang *et al* (1991)
elderly	epidem	pollution		10 yr	Diabetes, cardiovascular, lung cancer	Frank and Tankersley (2002)

Table 1.3 Combined effect of SO_2 and other air pollutants on humans and other species

Species, Subjects	Research	SO_2 concentration	Units	Duration	Effect	Reference
Young nonsmokers	trial	SO_2 0.36 Ozone 0.36	ppm	2 h	↑ absorption of ozone	Rigas *et al* (1997)
Hypersensitive bronchial system	trial	SO_2 0.5 NO_2 0.25	ppm	30 min	NO_2 ↑ bronchial effect of SO_2	Jorres *et al* (1990)
rats	trial	SO_2 4.0 + carbon particles	ppm	16 h/day for 11 months	Particles ↑ hyperplasia of pulmonary endocrine cells	Ito *et al* (1997)

Table 1.4 Uses of SO_2 in research to create models for human airway diseases

Species	SO_2 concentr	Units	Duration	Reference
Rats	600—700	ppm	3 h/day for a period of 30 h	Knauss *et al* (1976)
Rats	236 +/- 14	ppm	5 h/day, 5 d/wk for 7 weeks	Sweeney *et al* (1995)
Rats	230	ppm	5 h/day for a period of 1 day to 5 weeks	Farone *et al* (1995)
Rats	200	ppm	6 h/day for 5 d/wk for a period of 6 weeks	Kodavanti *et al* (2000)
Beagle dogs	500	ppm	2 h/day, 5 d/wk for a period of 16-21 weeks	Richards (1983), Greene *et al* (1984), Greene *et al* (1987)
Ferrets (*Mustela putorius furo*)	500	ppm	3 h/day, 3 d/wk for a period of 12 weeks	Miller *et al* (1985)

Table 1.5 Effect of SO_2 on biochemical and hematological parameters in various species

Species, Subjects	Research	SO_2 concentrations	Units	Duration	Effect	Reference
rats	Trial	0.87	ppm	24 h	Sulfhemoglobin ↑	Baskurt (1988)
humans	Epidemiological (=0.08 ppm)	200	$\mu g/m^3$	13 days	Cardiovascular	Peters *et al* (1997)
guinea pigs	Trial	400	ppm	3 h/day for 28 days	ALP ↑	Suchankova *et al* (1997)
Field mice	Pollution				leukocyte number ↑	Gorriz *et al* (1996)
passerine birds	pollution				erythrocyte count ↓	Llacuna *et al* (1996)
cynomolgus monkeys	Trial	0.1 & 5.0	ppm	78 wk	no	Alarie *et al* (1975)
guinea pigs	Trial	0.1 & 5.0	ppm	52 wk	no	Alarie *et al* (1975)
guinea pigs	Trial	0.13; 1.01;5.72	ppm	12 month	no	Alarie *et al* (1970)
Young rats	Trial	10	ppm	1 h/day, 7 d/wk, 6 wk	Vitamin C ↓ Ceruloplasmin ↓	Gumuslu *et al* (2000)
humans	Epidemiological				WBC count ↑	Schwartz (2001)

Table 1.6 Monoclonal antibodies (Mab's) as markers for identification of bovine peripheral blood leukocyte subpopulations

Bovine Mab's	Mab's	Subpopulations	Relationship between markers	References
BoT2	CD2	Mature T lymphocytes	$CD3^+$ $CD2^+$ $CD21^-$ $CD14^-$	Baldwin *et al* (1988); (Davis *et al* (1988)
BoT3	CD3	T lymphocytes	$CD4^+$ $CD8^+$ $WC1^+$	Davis *et al* (1993)
BoT4	CD4	"helper" T lymphocytes (Th)	$CD3^+$ $CD4^+$ $CD8^-$	Baldwin *et al* (1986); Howard *et al* (1989); Morrison *et al* (1994)
BoT8	CD8	Cytotoxic T lymphocytes (Tc)	$CD3^+$ $CD4^-$ $CD8^+$	Ellis *et al* (1986); MacHugh and Sopp (1991)
CD25	CD25	IL-2	$CD4^+$ $CD25^+$	MacHugh *et al* (1993)
TcR1	TcR1	αβ-T lymphocyte receptors	$CD4^+$ $TcR1^+$ $CD8^+$ $TcR1^+$	Wyatt *et al* (1994)
WC1	n/a	γδ-T lymphocyte receptors	$CD3^+$ $WC1^+$	Wijngaard *et al* (1992); Wijngaard *et al* (1994)
B cells	CD21	B cells	$CD3^-$ B $cells^+$	Sopp and Howard (1997)
CD14	CD14	Monocytes	$CD3^-$ $CD14^+$	Sopp *et al* (1996)

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Chapter 2

SULFUR DIOXIDE HEAD-ONLY EXPOSURE SYSTEM DESIGNED FOR CATTLE

INTRODUCTION

Animals and humans may be exposed to various atmospheric contaminants dispersed into the environment as a result of human activities. Studies of the effects of airborne agents on animals and man dates back well over a century. Various inhalation exposure systems have been developed to study the toxicity of noxious airborne substances (Phalen *et al* 1984). Early inhalation studies with small laboratory animals dating back to 1865 are described in review prepared by (Fraser *et al* 1959). The equipment included a simple wooden chamber, a gasometer to record rate of airflow that was driven by water displacement, and a manometer to sense pressure in the chamber. Modern inhalation technology now includes mass flow controllers continuously monitored by computer-based flow and pressure instrumentation along with analyzers to determine concentrations of gas components. Occupational health and safety and environmental guidelines for inhalation studies requires the establishment of a safe working environment for laboratory personnel and prevention of undesired discharge of pollutants to other animals not involved in the study (Dorato and Wolff 1991).

Sulfur dioxide (SO_2) is a major air pollutant, which is present in low concentrations in urban areas and in higher concentrations in certain work environments (ACGIH 1996). It has been suggested that the health of the cattle might be affected by breathing air polluted with SO_2 and other gases generated by fossil fuel recovery plants located in close proximity to farms (Coppock *et al* 1996). Controlled studies do not appear to exist on the toxicology of SO_2 in cattle. Since we did not find any recent reports on inhalation exposure systems for cattle, an inhalation exposure system was assembled to expose cattle to SO_2.

Three basic types of inhalation exposure systems exist that differ in how the test material is delivered: 1) static, with no airflow in the chamber; 2) recirculating, with a closed loop, and 3) dynamic, which has a single-pass flow-through system. Additionally, the inhalation systems can be classified into groups by the way an animal or human is

exposed to a toxic substance: 1) whole-body, 2) head-only, and 3) nose- or mouth-only methods (Phalen 1976). Chambers have been designed in a variety of sizes and shapes (MacFarland 1983), (Phalen 1976, (Phalen *et al* 1984, Nordstrom 1975) to suit specific species and conditions. Beerwinkle and Oehler (1982) designed a large-animal muzzle-only inhalation exposure system in which the principles of concurrent flow spirometry (CFS) were used. The system was completely enclosed and operated under a slightly negative pressure. Under these conditions, the system was used only for 2-hour exposure durations. Hays (1972) described a head-only inhalation system for exposing dairy cows to hydrogen sulfide (H_2S) for a period of up to 21 days. The hood was continuously ventilated with atmospheres with hydrogen sulfide (H_2S). Hydrogen sulfide concentration was measured in samples taken from the exposure hoods or the exhaust flow using a Kitagawa detector tube and syringe (Hays 1972).

The objective of this study was to test the hypothesis that cattle exposed to atmospheres with SO_2 would show physiological and toxicological responses in a concentration-dependent manner and that cold environment temperature would exacerbate the effects of SO_2. A head-only inhalation exposure system for cattle was designed with modifications of the system described by Hays (1972). Our exposure system incorporated computer-regulated mass flow controllers and continuous on-line analysis of atmospheres with SO_2.

MATERIALS AND METHODS

Inhalation exposure system for cattle

The inhalation system had to provide accurate amending of atmospheres with SO_2, be comfortable for the cattle, and meet occupational health and safety standards. There was also a requirement to have the experimental cattle housed in a temperature-controlled chamber, and achieve restraint of the animal in a comfortable manner during the exposure interval. All cattle (Simmental crossbred steers) used in these experiments were halter-trained for leading and were accustomed to the stanchions and hoods. The system consisted of hoods (Figure 2.1) each with a flexible, but airtight collar of appropriate length (for about 100 cm) that formed an air-tight fit around the neck and

permitted the animal limited freedom of movement (standing, lying, eating, drinking water, etc.). The hood was a modified version of a hood and used routinely at the University of Alberta for recording oxygen consumption of cattle in metabolism experiments (Young *et al* 1975). The training regime showed that cattle, that have been previously halter-broken, readily adapted to the hoods.

The modification of the hood was to have the air pressure inside the hood negative to the temperature-controlled chamber, and the air pressure in the temperature-controlled chamber negative to the rest of the building. This arrangement ensured that, if any leakage occurred from the hood, the gas would leaked into the temperature-controlled chamber and would subsequently be vented out of the building. It was possible to accommodate two animals per room by placing two hoods into each temperature-controlled chamber. The overall dimensions of each hood include a length of 76.5 cm, a width of 76.5 cm, and a height of 165.6 cm, with an approximate volume of 1000 L. Construction materials include an angle iron frame to which were attached sheets of standard white puck boards (Johnston Industrial Plastics Limited, Edmonton, AB, Canada). The hood has a sliding side door with a Plexiglas window for easy access and observation. All seams were sealed with a silicon caulking, and the door tightly closed against a rubber gasket. Each hood has an oval opening at one end that was about 115 cm by 37 cm with a tightly attached polyvinyl fabric that tightened snugly around the neck of the steer with a drawstring. The hoods were continuously ventilated using two pumps (Air blower with Tech TEFC motor, 1189 L/min, Cole-Palmer E-07047-10, Labcor technical sales, Quebec, Canada), one to force air into the chamber and another to draw air from the chamber. Using the two pumps set at slightly different rates allowed for a small negative pressure to be maintained in the hood during SO_2 exposure. The inlet port and gas diffuser were located in front of the chamber at a position below the nostrils of the animal and the exhaust port was located at the top of the hood. To prevent pressure surges due to a "bellows effect" when the animal moved forward or backwards in the chamber, a 30 cm diameter opening was made in the top panel and covered with a durable 10 L plastic bag. The plastic bag, as well as the polyvinyl fabric was held in place by puck board gaskets bolted around the openings and edges sealed with silicone.

Safety considerations for inhalation systems

The exposure hoods and animals were inside a sealed, temperature-controlled chamber with ventilation system that was isolated from the main building air handling system. A pressure differential of -2.54 to -5 cm of H_2O was maintained between the hood and the temperature controlled chamber to ensure that air leakage would be into the exposure hood. Any small leakage of SO_2 would be rapidly diluted by the chamber ventilation system. Individual protection for personnel, consisting of respirator, goggles, rubber gloves and boots, and personal SO_2 gas monitor with alarm (T-80 with SO_2 sensor 0-150 ppm, lithium battery, part 1810-2318-0005L, Safety Supply Division, Edmonton, Alberta, Canada), were provided in case of a SO_2 emergency.

Regulation of gas administration

Amending the atmospheres with SO_2 was regulated with mass flow controllers and was continuously monitored using SO_2 analyzers. A commercially purchased cylinder filled with 100% SO_2 (Matheson Gas Products Canada, Edmonton, Alberta, Canada) and supplied with pressure/reducer regulator was connected to the mass flow controller (MFC) (Brooks Redwood flow, USA) by means of a stainless steel connector pipe (~0.63 cm). The pure SO_2 was mixed in the air stream in a 1-inch polyvinyl chloride (PVC) pipe where it is diluted with filtered air to the preferred concentration set on the MFC (Figure 2.2). An automotive type air filter was used to filter the intake air. The air pressure in the air-supplied pipe was maintained at 2 centimeters of water column below the atmospheric. The SO_2 flow meter (Matheson, Modular Dyna-Blander, model 8250) down stream regulated the concentration of the gas mixture and matched the flow of SO_2 to maintain the air mixture at the specified concentration level. A T-pipe located in the temperature-controlled chamber permitted distribution of the gas mixture to one or two "head exposure" hoods with the airflow rate in each hood in the range of 300- 400 L/min. Atmospheres with SO_2 entered the front of the hood approximately 0.5 m above the floor. Airflow rates were sensed by a Pitot Tube Sensor (Pitot tube 0-5"H-20, nominal line size 1" plastic tubing, FPT-6110, Omega engineering, Quebec, Canada) and the differential

pressure was displayed on an LCD (display) indicator for quick reference. A cut off valve operated by a solenoid was used to stop the SO_2 flow any time that the air supply stopped flowing or if electrical power was interrupted. A fluorescent gas analyzer (model 8850, Monitor Labs Inc, USA) was used to determine the levels of SO_2 in the hoods. The SO_2 concentration was measured and recorded continuously on a chart recorder (OmniScribe recorder, Houston Instruments, Graphic division of Bausch and Lomb) and on the LabTech data acquisition and process control software (Laboratory Technologies Corporation, Wilmington, MA, USA). Before every SO_2 exposure, the analyzer was calibrated with fresh atmospheric air for baseline and standard SO_2 calibration gases of 5.3 and 44.8 ppm diluted with nitrogen (Matheson Gas Products Canada, Edmonton, Alberta, Canada).

Determination of the concentration of SO_2 in the hoods

When more than one hood was ventilated with SO_2, valves were installed so each exposure hood could be sampled in sequence (Figure 2.2). Plumbing for air monitoring of oxygen was also installed as previously described by Young *et al* (1975), (Figure 2.3). The system was initially tested without animals and subsequently with animals using atmospheres containing 1, 5, and 20 ppm of SO_2.

The Improvement of the existing inhalation system

Use of the hoods in studies in cattle on the effects of atmospheres with H_2S have been previously described (Hays 1972). Our first prototype involved one hood for control air and one hood for air with SO_2. In subsequent experiments, the system was modified to accommodate two SO_2 exposure and two control animals simultaneously, and eventually to accommodate four SO_2 exposure and four control animals simultaneously. The ability to expose concurrently two steers to SO_2 air and two steers to control (atmospheric) air required the use of two chambers. The air with SO_2 was distributed from a single mixing device into two exposure hoods, located in the same temperature-controlled chambers. To obtain representative samples of air from each hood we switched from one hood to

another every 20 min by using a hand-operated two-way valve located on the sampling line. Two control animals were accommodated in an adjacent temperature-controlled chamber. This allowed us to double the efficiency of the system and expose larger number of animals to SO_2 or clean air. Eventually, the system was modified to allow simultaneous exposure of four animals to SO_2 and four control steers to clean air delivered to another four hoods located in a different environmental chamber. It was decided to increase the capabilities of the system by connecting four hoods to a single gas mixing and distributing unit. Air with SO_2 was delivered into two hoods located in the "warm" (+15°C to + 17°C) chamber, and into two hoods located in the "cold" (-15°C to -17°C) chamber. In order to control the SO_2 concentration in each hood an automatic computerized switching aggregate was installed. The experimental head-only exposure hoods were designated as A, B, C, and D. Every 5 min in the repeating continuous order, the tested air from the hoods was passed in sequence through the SO_2 analyzer. As a part of the electronic set up with the aim of eliminate the carry-over effect of a previous gas concentration, the sampling line was consistently flushed with clean air for 1 min before switching to sampling of the next hood. Control animals housed in two other chambers with temperature conditions similar to the experimental ones were exposed to clean air in similar hoods. Atmospheres exhausted from the chamber were diluted with air and filtered with high-efficiency activated charcoal filters.

RESULTS

For animal exposures, the mean SO_2 concentrations during animal exposures to atmospheres with 1, 5, and 20 ppm of SO_2 in experiment 2 when 2 hoods only were used (23, 9 and 8 trials/exposure days) and in experiment 3 (10, 7 and 7 trials/exposure days) when 4 hoods were used are shown in tables 2.1 and 2.2 respectively. There was no significant difference ($P > 0.05$) between SO_2 concentrations in hoods A and B regarding all three tested concentrations of SO_2. When all 4 hoods were used there was no significant difference ($P > 0.05$) in the concentrations of SO_2 between hoods A and C at all 3 levels of SO_2; however, concentrations in hoods B and D were significantly different ($P < 0.05$) from the 2 other hoods and from each other at 1 and 5 ppm of SO_2. Hoods B

and D were not significantly different ($P > 0.05$) from each other at 20 ppm of SO_2 but they had significantly lower ($P < 0.05$) concentration of SO_2 compared to hoods A and C (Table 2.2).

DISCUSSION

The continuously ventilated head-only exposure hoods provided a convenient and repeatable inhalation exposure system for cattle. It was shown that the metering and mixing system accurately diluted the atmospheres with SO_2 and delivered these atmospheres to the exposure hoods. By using an expansion bag on the chamber, surges of air with SO_2 did not occur with animal movement. Maintaining a negative atmospheric pressure within the chamber ensured that atmospheres with SO_2 did not escape from the chamber into other areas of the laboratory. Comparison of mean SO_2 concentrations among the hoods indicated that to control all three levels of SO_2 was easier when only two hoods were functional. When four hoods were in use, there was a significant difference between pairs of hoods at 20 ppm of SO_2, and between three hoods when the concentration of SO_2 became smaller.

In order to avoid the possibility that steers were not evenly exposed to SO_2 due to variations of SO_2 concentrations in each hood, the animals were rotated among the hoods. Thus, it was possible to achieve uniformity and minimize variation of the administered SO_2 concentration when gas was distributed between two or among four hoods. This is consistent with the study outcomes performed by Yeh *et al* (1987) on rats. The rotation of the animals were also practiced in a study with a multi-leveled inhalation exposure chamber for rats designed by Griffis *et al* (1981). In the latter study, animals housed on one side of the chamber had lung burdens 8-11% greater than those on the opposite side. The authors recommended the rotation of animals among the chamber's six tiers to ensure equal treatments for each group of animals.

In the experiments with SO_2, the steers well tolerated the neck collar that was attached to the opening in the exposure hood or the overall hood restraint. The collar was made from polyvinyl fabric that is suitable for the inhalation purposes. Strong material did not allow gas to transpire through and, at the same time, the fabric was pliable and

easy to handle. This collar formed a leak-free seal between an SO_2 exposure hood and the temperature-controlled chamber. A similar collar was used for sealing rodents in a partial body plethysmograph (Esparza *et al* 1979). The length of the collar and the size of head opening allowed the steers to stand, to lie, to eat and to drink water. In spite of the limitations imposed on the steers, their tolerance to restraint in the stall and the head hood was good, in agreement with other reports. Bolinger *et al* (1997) conducted a study on Holstein cows, which were restrained in self-locking stanchions and headlocks, for approximately 4 hr/day. In this study in dairy cows the tested parameters such as milk production, dry matter intake, plasma cortisol concentrations and the ratio of neutrophils to mononuclear cells were not affected by the restraint (Bolinger *et al* 1997). The use of self-locking stanchions did not appear to affect substantially the overall wellbeing of the cows. To minimize possible stress-effects of restraint on the SO_2 study cattle, the animals were trained prior to the experiment to be accustomed to headlock stanchions and the exposure hoods. The importance of prior training has been suggested by previous studies including those performed on rats (Rothenberg *et al* 1995).

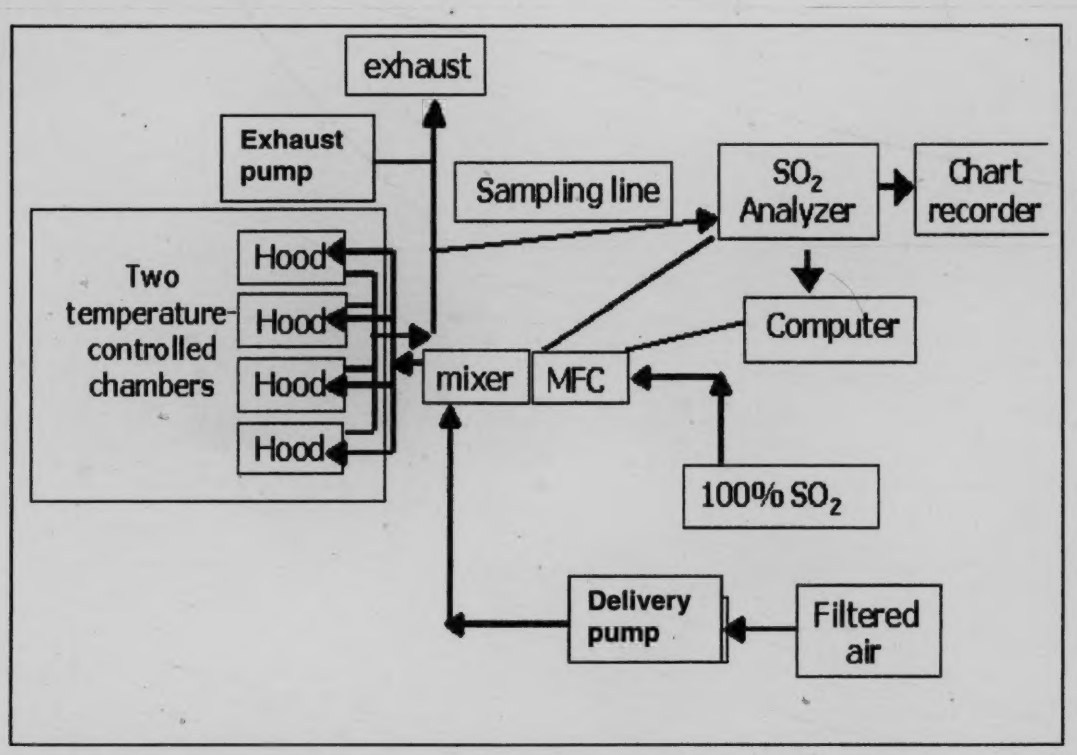
CONCLUSION

The head-only dynamic flow exposure system designed for this study appeared to be a dependable technique to provide precise and well-controlled exposure of large animals such as cattle to SO_2 gas at pre-determined concentrations ranging from 0 to 20 ppm despite the variations between the certain hoods. It is recommended to rotate animals among the exposure hoods to eliminate any uneven exposures.

Figure 2.1 A head-only exposure hood for cattle

This photograph shows the head-only exposure hood which is located in the temperature-controlled chamber. The steer is in his personal stall. The hood had a flexible but airtight collar that allowed the animal to stand, lie down, eat and drink water. A sliding door with a Plexiglas window can be seen at the side. An expansion bag and an exhaust opening, which are not seen on this photograph, are located at the top of the hood.

Figure 2.2 A simplified diagram of the final version of a head-only SO_2 inhalation exposure system for cattle



Four exposure hoods were located in two temperature-controlled chambers. Concentrated SO_2 gas ("100% SO_2") was diluted with filtered air to the desired concentration in a mixing chamber ("mixer") and administered to the hoods. Delivery and exhaust pumps maintained the airflow through each hood at a rate of 300 to 400 L/min. Samples of the air from each hood ("sampling line") were monitored in sequence by an SO_2 analyzer. The results were recorded on a chart recorder and a computer. The desired SO_2 concentration was set on mass flow controller (MFC).

Figure 2.3 SO_2 mixing and control system

Table 2.1 Concentration of SO_2 (ppm) in inhalation exposure hoods A and B (Lsmeans), 23, 11 and 8 for 1, 5 and 20 ppm, respectively

Intended concentration of SO_2	Actual concentration of SO_2 (Lsmean ppm)			
	Hood A	Hood B	Mean [SO_2] at each level of exposure	SEM
1 ppm	1.00	0.98	0.99	0.02
5 ppm	5.12	4.98	5.05	0.08
20 ppm	20.14	19.7	19.92	1.87

SEM - standard error of the mean

Table 2.2 The actual concentration of SO_2 (ppm) in inhalation exposure hoods A, B, C, and D (Lsmean), n = 10, 7 and 7 for 1, 5 and 20 ppm, respectively

Intended concentration of SO_2	Actual concentrations of SO_2 (Lsmeans ppm)					
	Hood A	Hood B	Hood C	Hood D	[SO_2] at each level of exposure	SEM
1 ppm	1.00[a]	0.84[b]	1.00[a]	0.94[c]	0.94	0.02
5 ppm	5.09[a]	4.87[b]	5.14[a]	4.71[c]	4.95	0.07
20 ppm	20.15[a]	19.07[b]	20.12[a]	18.84[b]	19.54	1.62

a, b - different letters within the row are statistically different, $p < 0.05$

SEM – standard error of the mean

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Chapter 3

RATE OF RESPIRATORY ACTIVITY IN PERIPHERAL BLOOD NEUTROPHILS DURING EXPOSURE CATTLE TO VARIOUS CONCENTRATIONS OF SULFUR DIOXIDE

INTRODUCTION

Sulfur dioxide (SO_2) is a waste product in the troposphere resulting from the burning of fossil fuels and geological phenomena. Controlled inhalation studies on humans and laboratory animals showed that SO_2 targets the lungs, decreases pulmonary function, and SO_2 has been implicated as a causative factor in bronchitis (Koren 1995, Krasnowska *et al* 1998) and bronchoconstriction (Koren 1995). Epidemiological studies linking cattle productivity with waste substances in air have shown that these waste products have an effect on the productivity of cattle (Scott, 1998) suggesting that waste products in the troposphere have systemic effects on the health of cattle.

To our knowledge, there have been no reports on the effects of SO_2 inhalation on immune cell function in humans and animals. Blood neutrophils, or polymorphonuclear leucocytes, are part of the innate immunological defense against pathogens (Kuby 1997). Neutrophils are the most actively migrating immunocytes and are active in acute inflammatory responses during which they migrate to the site of infection. To kill pathogens, neutrophils produce hydrogen peroxide (H_2O_2) and free radicals (NO) in a process known as oxidative burst (Kabbur *et al* 1997; Li 1999).

To assess the effect of SO_2 exposure on innate immunity of cattle we studied the oxidative function of blood neutrophils by measuring the production of oxidative compounds by intact neutrophils and those stimulated with phorbol myristate acetate (PMA). The hypothesis tested was that SO_2 may affect neutrophil function as reflected by changes in the rate of oxidative burst activity of bovine neutrophils. Cold stress may have interactive effects with SO_2 on neutrophil function in cattle.

The objective of the study was to identify concentrations of SO_2 that may have an impact on neutrophil function in cattle.

MATERIALS AND METHODS

This project was conducted at the large animal Metabolic Unit at the Edmonton Research Station of the University of Alberta. To perform the SO_2 inhalation study, we used an inhalation exposure system, described in Chapter 2. The elements of this exposure system were a head-only exposure hood with controlled air-flow, a mass flow controller of SO_2, and an SO_2 analyzer, all designed with emphasis on occupational safety. The experimental procedures used were approved by the Animal Policy and Welfare Committee at the University of Alberta and animals were cared for under the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

A series of three experiments was performed with a total of 30 growing crossbred Simmental steers. In all three experiments the steers were housed in a thermo-neutral environment (temperature +17 to +20°C) in individual pens (size 3.1 by 3.7 meters) located inside a well-ventilated barn. The pens were supplied with automatic water bowls and an automated waste cleaning system. The floor of each pen was bedded with "wood shavings" which were changed two times per week. The animals were fed similar rations consisting of 60% roughage (alfalfa cubes), 39.7% of concentrate (barley grain) and 0.3% of vitamin/mineral supplement. Feed allowances in each experiment were provided as some multiple of maintenance, estimated from metabolic body weight and metabolizable energy content of the feed (NRC 1996). The composition and the nutrient content of the diet fed to the steers are listed in Tables 3.4 and 3.5. Water was provided *ad libitum* for all animals. On the days free from the SO_2 exposure, the steers were routinely fed a complete ration in their pens at 8:30 a.m. On the exposure days all the concentrate and the forage cubes (minus 1 kg) were placed into each SO_2 exposure hood at 8:30 a.m. for the steers. The steers were fed the remaining 1 kg of forage cubes in their pens after the end of the SO_2 exposure procedure at 3:30 p.m. The animals were adapted to this type of feeding arrangement during the training period.

The SO_2 gas flow was stopped at 2:30 p.m., however the animals remained in the exposure hoods for one extra hour until the concentration of SO_2 reduced to 0 ppm, and it was safe to open the hoods. Prior to the experiments, the steers were halter-trained and

accustomed to the individual cattle stanchions with the continuously ventilated head-only exposure hoods.

The steers were observed daily for their behavior and feed and water intake. Body weight, rectal temperature, heart rate, thorax auscultation, and color of mucous membranes were documented twice a week. Hematological and serum biochemistry analysis were routinely performed once a week to monitor the overall health status of the steers.

Experiment 1 *(exposure to 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Two steers 18 months of age, with initial body weights of 635 kg and 615 kg, were used in this experiment. These animals were fed ration at a level calculated to provide approximately 1.6 times maintenance. This experimental procedure was performed in a thermo-neutral environment (+17 to +20 °C). Both steers were sequentially exposed to air with 5 ppm of SO_2 for a period of 7 days and with 20 ppm of SO_2 for 6 days. They had a 15-day recovery period after both, 5 and 20 ppm of SO_2 exposure.

Experiment 2 *(exposure to 0, 1, 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Twelve steers 6 to 9 months of age, and averaging 391 kg of initial body weight, were used in this experiment that was performed in a thermo-neutral environment (+17 to +20 °C). The animals were fed a ration at a level calculated to provide approximately 2.2 times maintenance requirements. A group of six steers was sequentially exposed to atmospheres with 1 ppm for 23 days, 5 ppm for 9 days, and 20 ppm of SO_2 for 8 days. A similar group of 6 control steers was exposed to ambient air ("0 ppm of SO_2") in hoods during the designated experimental exposure periods.

***Experiment 3** (exposure to 0, 1, 5, and 20 ppm of SO_2 in thermo-neutral and cold environments)*

Sixteen steers, 12 to 14 months of age, and averaging 534 kg of initial body weight, were used in this experiment. These animals were fed a ration at a level calculated to provide approximately 2.2 times maintenance energy requirement. A group of 8 steers was sequentially exposed to atmospheres with 1 ppm for 10 days, and then 5 and 20 ppm of SO_2 for 7 days at each level. This experiment was carried out simultaneously in two different thermal environments: thermo-neutral (+17 to +20 °C) and cold (-15 to -17°C) designated as "warm" and "cold" respectively. The steers and hoods were in a temperature controlled chamber. These animals, unlike in previous 2 experiments, were subjected to 2 different treatments. A similar group of 8 control steers was exposed to ambient air ("0 ppm of SO_2") during the designated exposure periods.

Duration of the SO_2 exposure

In all three experiments, the animals were exposed to SO_2 or clean air for 6 hours per day during five consecutive days. Within experiment 2 and 3, the steers were randomly allocated among experimental and control groups.

***Experiment 4** (effect of vitamin E and selenium supplementation)*

This is a follow-up experiment that was conducted with the steers previously exposed to 1, 5 and 20 ppm of SO_2 to examine the potential of nutritional treatments to reduce the impact of SO_2 (if any present) and enhance recovery in cattle. The experiment was performed after the end of each 20 ppm of SO_2 exposure period and lasted 14 days. A total of 14 steers from experiments 2 and 3 were used in experiment 4. Seven steers were given a single intramuscular (*m. glutaeus medius*) injection of an emulsion of selenium-α-tocopherol (E-Sel injection, DIN 01940279, Citadel Animal Health, Edmonton, AB). The dose was calculated as 45 µl per kg of body weight providing supplemental vitamin E as dl-alpha-tocopheryl acetate (3.2 IU/kg) and selenium as sodium selenite (0.05 mg/kg). Another group of 7 control steers received no vitamin E and selenium supplementation.

Initial pre-exposure values

In order to obtain the animals' initial values for the tested parameters, as well as to evaluate the initial health status of the steers, a 14 day pre-exposure period was conducted at the beginning of each experiment. During this period [study days (-14) to (0)], in which a thermo-neutral and cold environments (where applicable) were maintained.

Sample Collection

Blood sampling

Blood samples were collected from the jugular vein 2x per week into vacutainer tubes filled with sodium heparin for neutrophil respiratory burst assays at 08:00. The blood samples were kept in the fridge at 4°C for 2 hours before the assay. Mitogen-induced neutrophil respiratory burst was measured as an index of peripheral blood neutrophils respiratory activity.

Neutrophil functional parameters

The chemicals for buffers and media were purchased from Sigma Chemicals (St. Louis, MO).

A flow cytometry neutrophil respiratory burst assay (Vowells *et al* 1995) was used to assess respiratory responses of neutrophils. Neutrophil activation tests *in vitro* were performed to determine the response of neutrophils to mitogen phorbol myristate acetate (PMA), purchased from ICN (Montreal, PQ, Canada). The assay involves lysis of erythrocytes in whole blood, collection of neutrophils, and diffusion of added a non-fluorescent fluorescein analogue dihydrorhodamine (DHR), purchased from Molecular Probes (Eugene, OR) into the cells, hydrolysis it into a new compound rhodamine 123, which becomes trapped within the cells. Production of H_2O_2 oxidizes the intracellular compound into a highly fluorescent one, which is determined by the flow cytometry system and displayed on the computer as mean channel fluorescence (MCF). The

oxidative product formation by neutrophils occurs as a graded response to membrane stimulation by PMA (Bass *et al* 1983).

Whole blood (400 µl) from each steer was added to propylene tubes filled with 4 ml of warm lysis buffer and gently inverted for 5 minutes. Cell pellet was collected by centrifugation for 5 minutes at 1500 rpm, washed once with 4 ml of warm wash buffer and again centrifuged. Cells were resuspended in 400 µl of wash buffer and 1.8 µl of DHR (29 mM in DMSO) was added to each reaction tube and incubated in a water bath (37°C) for 5 minutes. From the tube (0 minutes) it was removed 100 µl and transferred into a immunofluorescence polypropylene Falcon tube (Fisher Scientific, Edmonton, Alberta, Canada). PMA solution (3.2×10^3 nM) (100 µl) was added to the remaining cells and incubated in the water bath for additional 5, 10 or 15 minutes after which time an aliquot was transferred into immunofluorescence tube and immediately placed on ice to stop the reaction (Pratt *et al* 2002). Wash buffer (100 µl) was added to the control tube (0 minutes), which was also kept on ice. Within the next hour the oxidation reaction was quantified by flow cytometry using a 488 ηm line for excitation with collection occurring at 525 ηm. The data was analyzed using CellQuest software (Becton Dickenson). Combined measures of forward and side light scatter were used to discriminate neutrophils from the remaining leukocytes, red blood cells and cell debris. Non-fluorescent parameters were collected (mean forward scatter and mean side scatter) to estimate changes in size and granularity (measures of activation) after 5, 10 and 15 minutes. Mean channel fluorescence of gated neutrophils was measured at 0, 5, 10 and 15 minutes as an estimation of the oxidative burst.

Statistical Analyses

Two steers in sequence were exposed to air with SO_2 in experiment 1, therefore, they both were designated as "treated" animals. The results across the exposure periods [pre-exposure (0ppm), 5 ppm, after 5 ppm, 20 ppm and after 20 ppm] are presented as arithmetic means of 2 steers. The steers in experiments 2 and 3 were grouped in blocks involving similar treatments, maintenance, and control conditions since only a few animals could be exposed to SO_2 simultaneously. The research study was performed

using a factorial experimental design (treated versus control animals). For the purpose of statistical analysis, we considered animals exposed to 0 and 1 ppm, 0 and 5 ppm, 0 and 20 ppm of SO_2 as separate groups, and carry over effect of the treatments could not be determined. Data were analyzed using Proc GLM with fixed effect of treatments [SO_2 and control (no SO_2)], environment (warm and cold) where applicable, and two blocks. In the case of animal neutrophil respiratory burst the analysis of variance were performed with the changes from initial pre-exposure values for all groups of animals to account for individual animal differences. Least Square Means (Lsmeans) were estimated using the Pdiff option (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA).

RESULTS

Experiment 1

Neutrophil respiratory burst

The arithmetic means of mean channel fluorescence (MCF) are expressed as a ratio of MCF at 5, 10 and 15 min of the incubation with phorbol myristate acetate (PMA) to 0 min (the basal rate of neutrophil respiratory activity - no incubation with PMA). The measurements of the respiratory activity were performed at 0, 5 and 20 ppm of SO_2 exposure levels and the oxidative burst at each level of SO_2 exposure (5 ppm, "after 5 ppm", 20 ppm and "after 20 ppm") was compared to 0 ppm of the SO_2 exposure. The neutrophil respiratory burst activity was decreased by 30%, 50% and 40% at 5/0, 10/0 and 15/0 stimulation indices, respectively at 5 ppm of SO_2 exposure level. At the resting period of 14 days after 5 ppm (no SO_2 exposure) the decrease in respiratory actitivity was the same as previous (30%) across all timepoints of the PMA incubation. Exposure steers to 20 ppm of SO_2 further decreased the respiratory burst by 70%, 70% and 60% across 5/0, 10/0 and 15/0 stimulation indices, respectively. The resting period of 14 days after 20 ppm (no SO_2 exposure) did not improve the oxidative activity of the neutrophils, and they remained decreased by 60, 70 and 70% compared to the pre-exposure period (0 ppm of SO_2) (Table 3.1).

Experiment 2

Neutrophil respiratory burst

Table 3.2 illustrates the changes in neutrophil respiratory burst activity at 0, 5, 10 and 15 minutes after stimulation with PMA. The MCF expressed as a ratio of MCF at 5, 10 and 15 min of the incubation with PMA to 0 min (the basal rate of neutrophil respiratory activity - no incubation with PMA). The measurements of the respiratory activity were performed at 1, 5 and 20 ppm of SO_2 exposure levels and the oxidative burst at each level of SO_2 exposure was compared to corresponding control values. The values obtained at the pre-exposure period (0 ppm) were used as a covariate in statistical analysis by one-way ANOVA. The basal level (0 min) of neutrophil respiratory activity was not affected ($P= 0.1$) by any level of SO_2 exposure. At 1 ppm of the SO_2 exposure level the stimulation indices at 5 min of 16 and 14 between control steers and steers, respectively, were not different ($P= 0.474$). The indices became significantly ($P < 0.01$) decreased by 38% at 10 min and by 17% at 15 min of PMA stimulation due to 1 ppm of SO_2. In the steers exposed to 5 ppm of SO_2 the stimulation indices were significantly decreased ($P< 0.01$) by 38% at 5 min, by 55% at 10 min and by 40% at 15 min compared to their corresponding controls. In the steers exposed to 20 ppm of SO_2 there were significant decreases ($P < 0.01$) of 56, 72 and 59% in the stimulation indices at 5, 10 and 15 min after stimulation with PMA (Table 3.2).

Experiment 3

Neutrophil respiratory burst

Table 3.3 shows the indices of 5, 10 and 15 min of the neutrophil respiratory burst activity after stimulation with PMA measured at 1, 5 and 20 ppm of SO_2 exposure in warm and cold environments. The basal level (0 min) of neutrophil respiratory activity was not affected ($P= 0.6$) by any level of SO_2 exposure in both warm and cold environments. The 5 min index was not affected by 1 ppm of SO_2 exposure ($P> 0.05$) in either environment. The results obtained in the warm environment were similar to those from the experiment 2. The stimulation index at 10 min was significantly ($P< 0.05$)

decreased by 42, 34 and 62% after exposure steers to 1, 5 and 20 ppm of SO_2 compared to their corresponding controls. The stimulation index at 15 min was significantly ($P< 0.05$) decreased by 37, 46 and 60% after exposure steers to 1, 5 and 20 ppm of SO_2 compared to their corresponding controls. In the cold environment, the 5 min stimulation index was significantly ($P< 0.01$) decreased after exposure of steers to 5 and 20 ppm of SO_2. However, at 10 and 15 min of the neutrophil stimulation with PMA the indices were not different ($P> 0.05$) between experimental and control groups of steers at any level of SO_2 exposure in the cold environment. There was a significant interaction between environmental temperature and SO_2 ($P= 0.007$). There was a non significant ($P= 0.09$) decrease in the 5 min index in steers exposed to 1 ppm of SO_2 compared to their corresponding controls in warm environment. These may be due to the fact that we were using only 4 steers per treatment in this experiment. The calculations of the power of the test presented on the page 86 suggest that n = 9 would be required to detect a significant difference at $P< 0.05$.

Experiment 4

Total neutrophil count

During the 2-week period following the end of SO_2 exposure, neutrophil numbers were significantly ($P< 0.05$) increased from 1590 cells/mm^3 in controls to 2513 cells/mm^3 SE= 183 (by 58%) in response to the vitamin E/selenium treatment.

Neutrophil respiratory burst

Figure 3.1 shows the neutrophil respiratory burst activity in control and vitamin E/selenium treated steers during the period following SO_2 exposure at the 20 ppm level. Even though neutrophil numbers were enhanced, there was no significant effect of treatment on the neutrophil functional responses to stimulation by phorbol myristate acetate (PMA) due to vitamin E/selenium treatment.

DISCUSSION

This study has demonstrated that the neutrophil respiratory burst activity measured at 10 and 15 min of stimulation with PMA was reduced after exposure of steers to 1, 5, and 20 ppm levels of SO_2 in a warm environment. However, the effect of SO_2 on neutrophil respiratory activity was modified by the cold environment as indicated by the interaction of SO_2 and cold treatments (P= 0.007). In each experiment, there was evidence for some suppression of mitogen-induced neutrophil respiratory burst activity. The *in vitro* studies of the intracellular effects of sulfite in human (Beck-Speier *et al* 1994) and bovine (Komarnisky *et al* 2000) neutrophils support our findings that SO_2 suppresses the neutrophil respiratory burst activity. In the cold environment, the suppression of neutrophil activation was limited to the 5 and 20-ppm exposure level, suggesting a moderating effect of cold exposure for this aspect of immunological function. However, there was no evidence that cold exposure enhanced neutrophil activation in control steers.

One of the ways that neutrophils kill pathogens is to generate reactive oxygen radicals by their respiratory burst activity (Constantin *et al* 1995). Respiratory activity is a measure of overall metabolic integrity of phagocytising neutrophils. This is a membrane-associated fenomenon (Deitch 1984). Neutrophils can oxidize dihydrorodamine 123 (DHR), which is a dye that is oxidized by generated free radicals to form the brightly fluorescent compound rhodamine 123 through the hexose monophosphate shunt by respiratory burst oxidase (Jackson 2000). The respiratory burst oxidase, or nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase, of neutrophils is a multicomponent enzyme that catalyzes the reduction of oxygen to superoxide anion ($O_2^{\cdot-}$) (Benna *et al* 1994). In phagocytes in the resting state the components of the NADPH oxidase are located in the cytosol (Grogan *et al* 1997) and are transferred to the plasma membrane when activated. Thus, the membrane plays an important role in formation of phagocytic vacuole (Valerius *et al* 1981). It was reported that SO_2 exposure increased lipid peroxidation in red blood cell membrane (Kucukatay *et al* 2003).

The implication of our current results is that the killing action of neutrophils may be compromised when cattle are exposed to SO_2 at levels ranging from 1 to 20 ppm. This could have a negative impact on the health of cattle exposed to SO_2 waste in the troposphere. The assumption is that the *in vitro* mitogen activation test mimics what happens to neutrophils normally in the intact animal when pathogens invade.

The mechanism by which neutrophil activation is suppressed in response to SO_2 exposure is not clear. The suppression might be indicative of a more subtle effect of prolonged stress induced by periods of intermittent SO_2 exposure. There are well-established links between stress and the immune system involving interactions between the nervous, endocrine, and immune systems (Kuby 1997, Elenkov *et al* 2000, Kohm and Sunders 2000). We may speculate that SO_2 through the process of lipid peroxidation (Kucukatay *et al* 2003) in the cell membrane inhibits the respiratory burst activity of blood neutrophils by affecting the link in a multi-staged pathway by which the respiratory burst oxidase catalyzes the NADPH-dependent reduction of oxygen to superoxide. However, further study is needed to confirm or reject this hypothesis. Whatever mechanism accounts for reduced immune function, potential health implications may exist as a result of exposure of steers to SO_2 waste, since neutrophils serve as a first line of the innate immune defense in many diseases and, in particular, play an important role in defense of cattle against infections (Van Oostveldt *et al* 2002). Cattle exposed to environmental SO_2 may be more susceptible to disease because of the apparent suppression of neutrophil activation. However, we have not had an opportunity to test this possibility by direct pathogen challenges.

Even though neutrophil numbers were enhanced following vitamin E/selenium treatment, there was no significant effect of the nutritive treatment on the neutrophil functional responses to stimulation by PMA. Only a non-significant trend towards increased activation in steers treated with vitamin E and selenium was noted. Despite the fact that the treatment with vitamin E and selenium compensated the reduction in plasma vitamin E during SO_2 exposure, this supplement did not significantly improve neutrophil activity in our steers. A beneficial effect was expected since Politis *et al* (1995) showed that vitamin E supplementation (5000 IU injection plus 3000 IU/day given orally) to cows prevented the drop in neutrophil superoxide production that occurs after parturition.

In our study either the single injection of 3.2 IU/kg of vitamin E/ Se at the start of the 14-day evaluation period was not a sufficient dose to have an effect or the suppression of neutrophil respiratory activity by SO_2 was not directly linked to vitamin E or selenium status of the steers. Neutrophils are the first line of defense against a microbial infection, and therefore, nutritional or other therapeutic treatment of SO_2 exposed animals might ultimately be important for health. It is apparent that further work is needed to evaluate respiratory function of neutrophils in animals that are exposed to SO_2 and are challenged by immunization to mimic infectious disease.

Table 3.1 Mean Channel Fluorescence (MCF) values as an index of respiratory activity in response to stimulation of peripheral blood neutrophils with PMA in steers exposed to 0, 5 and 20 ppm of SO_2. The measurements were also performed after 5 and 20 ppm periods of SO_2 exposure 15 days in duration each, 2 steers, experiment 1

The neutrophils were incubated with PMA at 0, 5, 10 and 15 min. The arithmetic means are expressed as a ratio of MCF at 5, 10 and 15 min to 0 min (basal rate)

SO2 level	Effect	Index 5/0	Index 10/0	Index 15/0
0 ppm		28	40	49
5 ppm		18	22	30
	decreased (by %) compared to 0 ppm	30%	50%	40%
After 5 ppm		19	29	34
	decreased (by %) compared to 0 ppm	30%	30%	30%
20 ppm		9	14	19
	decreased (by%) compared to 0 ppm	70%	70%	60%
After 20 ppm		10	12	16
	decreased (by %) compared to 0 ppm	60%	70%	70%

Table 3.2 Neutrophil respiratory burst activity, measured as mean channel fluorescence (MCF; % gated) by flow cytometry, in control steers and steers exposed to 1, 5 and 20 ppm of SO_2. Data are expressed as a ratio of 5/0 min, 10/0 min and 15/0 min of stimulation time with phorbol myristate acetate (PMA), n = 6, experiment 2

Ratio	Level of SO_2	Treatment		SEM
		Control[1]	SO_2	
5/0 min	1 ppm	16^a	14^a	1
	5 ppm	16^a	10^b	1
	20 ppm	16^a	7^b	1
10/0 min	1 ppm	56^a	35^b	4
	5 ppm	56^a	25^b	4
	20 ppm	58^a	16^b	4
15/0 min	1 ppm	60^a	50^a	5
	5 ppm	57^a	34^b	5
	20 ppm	54^a	22^b	5

a, b = Means within a row with different superscripts are different ($P< 0.05$)
SEM = standard error of the mean
[1] control = atmospheric air

Table 3.3 Neutrophil respiratory burst activity measured as mean channel fluorescence (MCF; % gated) at 0, 5, 10 and 15 min time after stimulation with phorbol myristate acetate (PMA) in control steers and steers exposed to 1, 5 and 20 ppm of SO_2 in warm and cold environments. Data are presented as ratio of means at 5/0 min, 10/0 min and 15/0 min of stimulation with PMA, n = 4, experiment 3

		Treatment				
		Warm		Cold		
Ratio	SO_2 Level	Control[1]	SO_2	Control[1]	SO_2	SEM
5/0 min	1 ppm	21^{a}	13^{*}	19^{a}	16^{a}	3
	5 ppm	27^{a}	13^{b}	24^{a}	12^{b}	3
	20 ppm	33^{a}	10^{b}	22^{a}	13^{b}	3
10/0 min	1 ppm	78^{a}	45^{b}	82^{a}	67^{a}	10
	5 ppm	73^{a}	48^{a}	62^{a}	45^{a}	10
	20 ppm	80^{a}	30^{b}	54^{ab}	42^{ab}	10
15/0 min	1 ppm	98^{a}	62^{b}	99^{a}	86^{ab}	14
	5 ppm	102^{a}	55^{b}	74^{ab}	50^{b}	14
	20 ppm	134^{a}	53^{b}	67^{b}	76^{b}	14

a, b = Means within a row with different superscripts are different ($P< 0.05$)
SEM = standard error of the mean
[1] control = atmospheric air
* SO_2 treatment differs from control in the warm environment, $P= 0.09$

*Calculations of sample size (P= 0.09): the procedure by Stein (Steel *et al* 1997)

$N = (t^2 * s^2)/ d^2$, where N is a sample size, $t = 2.015$ is a tabulated value for confidence level of 95% and degree of freedom (df) is 6, s is a standard deviation = 6, d is half width of the confidence interval that is $\mu_1 - \mu_2 = 21 - 13 = 8/2 = 4$.

$N = [(2.015)^2 * 6^2]/4^2 = 4.06*36/16 = 12.4 = 9$.
Sample size of 9 steers would make this difference significant at P= 0.05.

Figure 3.1 Neutrophil respiratory burst (MCF) in samples from non-supplemented (control) steers and steers treated with vitamin E and selenium following SO_2 exposure at 20 ppm level, n = 7, experiment 4.

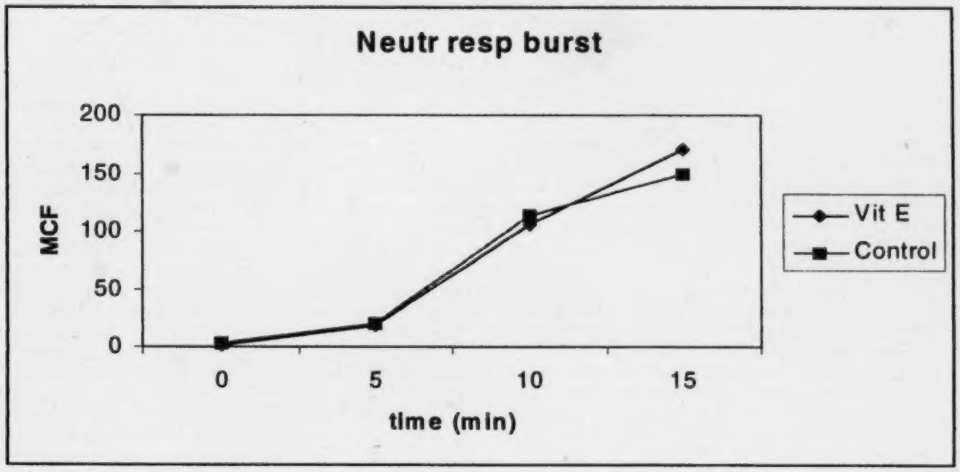

Table 3.4 Composition of the diet fed to steers in the SO_2 experiments

Ration component	% of DM
Alfalfa cubes	60.00
Barley grain	34.84
Canola meal	3.12
Canola oil	0.98
Calcium phosphate (16% Ca, 20.5% P)	0.09
Calcium carbonate (38% Ca)	0.58
Vitamin A, D, E and Rumensin*	0.10
Salt, Fortified, Champ**	0.16
Dynamate***	0.13
Total	100.00

* Vitamin/ Rumensin mix contained: ground barley 66.7 %, vitamins A, D, and E (IU/kg) 22.2% (Vitamin E = 1000 IU/kg of concentrate), rumensin 11.1%;

** Champion fortified salt contained: 95% salt, 38% sodium, iodine 150 mg/kg, cobalt 50 mg/kg, copper 3500 mg/kg, manganese 10,000 mg/kg, zinc 9000 mg/kg, and selenium 75 mg/kg;

*** Dynamate contains 22% sulfur, 18% potassium, and 11% magnesium

Table 3.5 Nutrient content of the diet for the steers, experiments1, 2 and 3

Feed name	DM %	NEM Mcal/kg	NEG Mcal/kg	Exp 1	Exp 2	Exp 3	Ca %	P %
				CP % of DM				
alfalfa cubes	90	1.1	0.55	16.7	15.2	14.8	0.9	0.15
Barley grain	88.5	2.03	1.37	12.8	13.6	13.1	0.07	0.38
Canola meal	91.9	1.63	1.03	39.2	39.2	39.2	0.75	1.26

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Chapter 4

PLASMA CONCENTRATIONS OF VITAMIN E AND VITAMIN A AND SERUM CONCENTRATIONS OF SELENIUM AND ACUTE PHASE PROTEINS IN CATTLE EXPOSED TO DIFFERENT LEVELS OF SULFUR DIOXIDE

INTRODUCTION

The air pollutant sulfur dioxide (SO_2) is a respiratory irritant, which may induce pulmonary damage and also oxidative stress (Menzel 1972). Sources of SO_2 in Alberta mainly come from human activities, such as combustion of sulfur-containing fossil fuels, coal-fired electrical generation, emissions from sour gas flares, sulfur recovery plants, recovery of oil from tar sands (Scott 1998). Oxidative stress arising from pollutants such as SO_2 may increase the demand for antioxidants including selenium (Se) and vitamin E, and possibly other nutrients such as vitamin A (Stephens *et al* 1983).

The dietary requirement for Se by cattle is 0.3 – 1.0 ppm dry matter (Puls 1994) for maintaining performance of immune and reproductive systems. With adequate dietary intake serum will contain about 30% of whole blood Se (Puls 1994). The dietary requirements for Se and its metabolism are influenced by many nutrient interrelationships, including its interactions with sulfur, lipids, vitamin E, proteins, amino acids, and several microelements (Ammerman and Miller 1975).

Selenium deficiency is responsible for Zenker type muscle degeneration in calves, lambs, and foals in the prenatal and postnatal stages of development (Bostedt and Schramel 1990). Postnatal nutritive muscular dystrophy, however, is attributed to both acute Se and vitamin E deficiency in basic feed or impaired plant absorption of Se as a result of antagonistic elements, such as sulfur (Bostedt and Schramel 1990). The investigation of the effect of altered Se nutrition on arachidonic acid oxidation in immune cells demonstrated that the concanavalin A-stimulated lymphocyte proliferation was significantly lower in Se-deficient cows (Cao *et al* 1992). Studies on cattle concluded that dietary Se status, which in turn determines tissue Se concentration, plays an important role in the regulation of arachidonate metabolism in peripheral blood lymphocytes. This may be one of the biochemical mechanisms underlying the inhibition of lymphocyte

proliferation and the decrease in resistance to infectious diseases observed in Se-deficient animals (Cao *et al* 1992).

Vitamin E is a mixture of eight related compounds known as tocopherols that are synthesized by plants. The α-tocopherol molecule is the most potent of the tocopherols. Vitamin E is absorbed from the intestines packaged in chylomicrons. It is delivered to the tissues via chylomicron transport and then to the liver through chylomicron remnant uptake. Due to its lipophilic nature, vitamin E accumulates in cellular membranes, fat deposits and other circulating lipoproteins (Basu and Dickerson 1996). Vitamin E acts as a natural antioxidant by scavenging free radicals and molecular oxygen. In particular vitamin E is important for preventing peroxidation of membrane polyunsaturated fatty acids in neurons (Tamagno *et al* 2000). The vitamins E and C are interrelated in their antioxidant capabilities. Active α-tocopherol can be regenerated by interaction with vitamin C following scavenge of a peroxy free radical (Lee and Dabrowski 2003). Alternatively, α-tocopherol can scavenge two peroxy free radicals and then be conjugated to glucuronate for excretion in the bile (Basu and Dickerson 1996).

The role of dietary interventions such as vitamin E and Se against tissue injury by oxidizing gases has been addressed by several investigators (Goldstein *et al* 1970), (Menzel *et al* 1972), (Elsayed *et al* 1983), (McMillan and Boyd 1982). It has been postulated that the antioxidant properties may be the result of vitamin E being preferentially oxidized by the oxidizing gas, thereby preventing the formation of lipid peroxides, or vitamin E may react with free radicals produced by lipid peroxidation (Dillard *et al* 1982).

Vitamin A (or Retinol) consists of three biologically active molecules: retinol, retinal (retinaldehyde) and retinoic acid (Wingerath et al 1999). Each of these compounds are derived from the plant precursor molecule, β-carotene. Ingested β-carotene is cleaved in the lumen of the intestine by β-carotene dioxygenase to yield retinal. Retinal is reduced to retinol by retinaldehyde reductase, an NADPH requiring enzyme within the intestines (Siegenthaler *et al* 1990). Plasma transport of retinoic acid is accomplished by binding to albumin (Basu and Dickerson 1996). Retinol is an essential nutrient which has many physiological effects throughout the body. It is stored in the liver and deficiency of the vitamin occurs only after prolonged lack of dietary intake. Vitamin A is necessary for

support of growth, reproduction, and maintenance of higher animals. In the absence of vitamin A, animals will stop to grow and eventually die (Frye et al 1991). It was suggested that beta-carotene plays a role in bovine health and reproduction (Chew et al 1983). *In vitro* assays that emphasize cellular components critical to the host defense system indicate that vitamin A is required for epithelial cell differentiation (Fisher and Placker 1987). It was also shown in rats that retinol may decrease the inflammatory response through inhibition of pulmonary macrophage function, thus resulting in decreased chemically-induced pulmonary injury (Sauer et al 1995).

Acute-phase proteins (APPs) are a large group of glycoproteins in the serum, which are synthesized by liver parenchymal cells and released into the bloodstream in response to a variety of stressors (Mehta 1977). Local inflammation, bacterial infection, endotoxin injection, neoplasia and thermal or mechanical injury all can induce release of APPs as part of the acute phase of the inflammatory reaction. The synthesis of APPs is thought to be stimulated by cytokines such as interleukins 1 and 6 (IL-1, IL-6), and tumor necrosis factor (TNF) (Godson *et al* 1995). Therefore, APPs are valuable parameters to monitor for assessment of animal health and are sensitive biomarkers for severe infection, inflammation, and trauma (Godson *et al* 1995). The biological functions of APPs are variable with some functioning as proteinase inhibitors, enzymes, transport proteins, coagulation proteins, and modulators of the immune response. However, all APPs appear to play a role in the restoration of homeostasis after injury, tissue necrosis, or infection. APPs such as serum amyloid A, haptoglobin, and alpha 1-acid glycoprotein (orosomucoid) have been identified as markers of inflammation in cattle (Horadagoda *et al* 1999). They would be expected to serve as indicators of tissue damage and inflammation due to SO_2.

Experiments were conducted with growing steers in controlled environment chambers to examine the effects of exposure to atmospheres with various concentrations of SO_2. The SO_2 levels were selected by reference to literature on humans and other animal species. An attempt was made to select levels close to, as well as above, those encountered in the environment near sources of SO_2 emission. Intermittent rather than continuous exposure was thought to more closely resemble occurrences in the field (Dorato and Wolff 1991).

***Hypothesis*:** SO_2 stress will decrease vitamin A, E and Se levels in blood of cattle exposed to SO_2 pollution due to increased metabolic demands. Cold stress, not unlike that which occurs during the winter months in Alberta, may have interactive effects with the effects of inhalation exposure to SO_2. Supplementation with vitamin E and Se will improve immune cell function in cattle affected by SO_2.

Objectives of the study were to determine whether atmospheres with SO_2 influences plasma concentrations of vitamin A, vitamin E and Se, and whether vitamin E and Se supplements will improve immune cell function in steers after SO_2 exposure.

MATERIALS AND METHODS

Four experiments were conducted in this study. For housing, feeding and management of the steers refer to Chapter 3.

Experiment 1 *(exposure to 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Two steers 18 months of age, with initial body weights of 635 kg and 615 kg, were used in this experiment. These animals were fed their ration at a level calculated to provide approximately 1.6 times maintenance. This experimental procedure was performed in a thermo-neutral environment (+17 to +20 °C). Both steers were sequentially exposed to air with 5 ppm of SO_2 for a period of 7 days and with 20 ppm of SO_2 for 6 days. They had a 15-day recovery period after both, 5 and 20 ppm of SO_2 exposure.

Experiment 2 *(exposure to 0, 1, 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Twelve steers 6 to 9 months of age, and averaging 391 kg of initial body weight, were used in this experiment that was performed in a thermo-neutral environment (+17 to +20 °C). The animals were fed a ration at a level calculated to provide approximately 2.2 times maintenance requirements. A group of six steers was sequentially exposed to atmospheres with 1 ppm for 23 days, 5 ppm for 9 days, and 20 ppm of SO_2 for 8 days. A

similar group of 6 control steers was exposed to ambient air ("0 ppm of SO_2") in hoods during the designated experimental exposure periods.

Experiment 3 *(exposure to 0, 1, 5, and 20 ppm of SO_2 in thermo-neutral and cold environments)*

Sixteen steers, 12 to 14 months of age, and averaging 534 kg of initial body weight, were used in this experiment. These animals were fed a ration at a level calculated to provide approximately 2.2 times maintenance energy requirement. A group of 8 steers was sequentially exposed to atmospheres with 1 ppm for 10 days, and then 5 and 20 ppm of SO_2 for 7 days at each level. This experiment was carried out simultaneously in two different thermal environments: thermo-neutral (+17 to +20 °C) and cold (-15 to -17°C) designated as "warm" and "cold" respectively. The steers and hoods were in a temperature controlled chamber. These animals, unlike in previous 2 experiments, were subjected to 2 different treatments. A similar group of 8 control steers was exposed to ambient air ("0 ppm of SO_2") during the designated exposure periods.

Duration of the SO_2 exposure

In all three experiments, the animals were exposed to SO_2 or clean air for 6 hours per day during five consecutive days. Within experiment 2 and 3, the steers were randomly allocated among experimental and control groups.

Experiment 4 *(effect of vitamin E and selenium supplementation)*

This is a follow-up experiment that was conducted with the steers previously exposed to 1, 5 and 20 ppm of SO_2 to examine the potential of nutritional treatments to reduce the impact of SO_2 (if any present) and enhance recovery in cattle. The experiment was performed after the end of each 20 ppm of SO_2 exposure period and lasted 14 days. A total of 14 steers from experiments 2 and 3 were used in experiment 4. Seven steers were given a single intramuscular (*m. glutaeus medius*) injection of an emulsion of selenium-α-tocopherol (E-Sel injection, DIN 01940279, Citadel Animal Health,

Edmonton, AB). The dose was calculated as 45 µl per kg of body weight providing supplemental vitamin E as dl-alpha-tocopheryl acetate (3.2 IU/kg) and Se as sodium selenite (0.05 mg/kg). Another group of 7 steers received no vitamin E and Se supplementation.

Initial values of the tested parameters

A pre-exposure period of 14 days in duration was conducted at the beginning of each experiment, in order to obtain the initial values for the tested parameters, as well as to evaluate the initial health status of the steers. During this period [study days (-14) to (0)], in which a thermo-neutral and cold environment (where applicable) were maintained in the temperature controlled chambers the steers were placed in hoods for 6 h per day and ventilated with fresh air.

Sample Collection

Blood sampling

Blood samples from steers in experiments 1, 2, 3 and 4 were obtained by venipuncture (external jugular vein) twice a week in the morning. Blood for serum was collected into blood tubes with a serum separator (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) The tubes were kept at room temperature for about 1 hour; and then centrifuged at 1000 g for 10 minutes, and the serum was transferred into 2 polypropylene tubes. The serum samples were stored at -80°C.

Blood for plasma isolation was collected into tubes containing sodium heparin anticoagulant (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). The tubes were centrifuges at 1000 g for 20 minutes, and the plasma was transferred into 2 polypropylene tubes. The plasma samples were purged with nitrogen in order to prevent oxidation of vitamins and stored at -80°C.

Alpha-tocopherol (Vitamin E) and Retinol (Vitamin A)

Vitamin E concentrations were determined in experiments 1, 2, 3 and 4; vitamin A concentrations were determined only in experiments 2, 3, and 4. Vitamin E as a single parameter was quantified by the same procedure as described below, only without addition of α-*trans*-retinol and retinyl acetate.

Extraction of retinol and α-tocopherol were performed by using the following extraction method of Nierenberg and Lester (1985). Stock solutions of all-*trans*-retinol and α-tocopherol were prepared in methanol and stored covered in foil, at -20°C. Standard dilutions were prepared in acetonitrile for each batch of samples with concentrations ranging from 1.7 to 10.2 mg/ml for α-tocopherol and from 1.6 to 9.6 mg/ml for all-*trans*-retinol. 120 µl of acetonitrile and 20 µl of retinyl acetate (2.23 mg/ml) were added to 400 µl of plasma. The samples were vortexed for 30 sec. The vitamins in plasma were extracted with 160 µl of solvent (butanol:ethyl acetate, 1:1), vortexed for 1 min, and precipitated with di-potassium phosphate (K_2HPO_4), vortexed for 30 sec. The organic layer was separated by centrifugation at 1000 g for 2 min and decanted into HPLC vials. 50 µl of this organic layer was injected into the high-pressure liquid chromatography (HPLC) apparatus. All samples were prepared in duplicate and under dim light to prevent possible oxidation of vitamins. All solvents used in the procedure were HPLC grade purchased from Fisher Scientific, Nepean, Ontario.

Vitamins E and A were identified and quantified in the plasma extracts by HPLC using a modified procedure by (Catignani and Bieri 1983). A Varian Vista 5500 liquid chromatograph combined with Varian 9090 autosampler (Walnut Creek, CA, USA) was used. The chromatograph was equipped with a 50 mm x 4.6 cm guard column containing Supelco LC-18 (Supelco, ON, Canada) reverse phase packing (20-40 µm), and the retinol and α-tocopherol in blood serum was separated on a 4.6 mm x 15.0 cm Supelcosil LC-18 (Supelco, Mississauga, ON) 5 micron reverse phase analytical column. Vitamin E was detected by a Shimadzu RF-535 fluorescence (excitation 295 nm and emission 330 nm) detector (Columbia, MD) and vitamin A was identified with UV detector (LCD spectroModel III, model 1204A) set at a wavelength of 325 nm. The mobile phase was 95:5 (acetonitrile: methanol) at a flow rate of 1.5 ml min^{-1}. Retention times were 2.7 min for retinol and 9.8 min for α-tocopherol, and the injection volume was 50 µl at ambient

temperature. The total run time was 12 minutes. Alpha-tocopherol concentrations were determined using an external standard method and retinol was determined using an internal standard method. Retinyl acetate was used as the internal standard (Sigma, St. Luis, MO).

The concentration of the analytes was calculated using a software system CLASS-VP version 4.2, Shimadzu Corp. (Shimadzu Scientific Instrument, Inc., Reverwood Drive, Columbia, MD, USA). The percent recovery of retinol after extraction was 95.5 ± 5.1% and α-tocopherol was 96.3 ± 4.5%.

Acute Phase Proteins (haptoglobin and orosomucoid)

Blood serum analysis for detection of the levels of acute phase proteins was performed only in experiment 3. The concentrations of haptoglobin and orosomucoid (alpha 1 acid glycoprotein) were determined by the radial immunodiffusion assay Kits purchased from Saikin Kagaku Institute Co., Ltd Sendai, Japan. There is a separate kit for haptoglobin and orosomucoid. Each test kit contains 10 test plates with 10 test wells in each plate. The procedure principle: A bovine serum sample is placed in an individual well, as the sample diffuses radially from the well into the agar gel plate which has been impregnated with anti bovine haptoglobin or anti bovine orosomucoid, a specific precipitin reaction occurs and a visible ring is formed. The area within this ring is directly proportional to the concentration of the haptoglobin or orosomucoid being measured.

For the haptoglobins serum samples were diluted with a 40mM L-cysteine solution (e.g. 0.1 ml of L-cysteine to 0.1 ml of serum sample); standards for the haptoglobin were not diluted. There was no dilution step for orosomucoid serum samples required, using a micropipette 5 µl of serum (orosomucoid) or 5 µl of diluted sample (haptoglobin) were applied to the appropriate plate. The plates were incubated in placed in a humidified atmosphere at 37 degrees C for 24 h. The plate was read on a plate reader (model AD001, provided by The Binding Site Ltd., Birmingham. Each plate had 2 standards and 8 samples for orosomucoid and 2 standards 7 samples and 1 control sample for haptoglobin. The precipitin ring measured for both standards were plotted on semi-logarithmic graph paper provided with the kit. A straight line was drawn through the points and was used as the reference curve to read of the unknown samples. A dilution

correction for haptoglobins for the serum samples was made. The final results are reported out in micrograms per microliter (µg/µl).

Selenium (Se)

Selenium level in bovine serum was identified and quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Perkin-Elmer Elan 5000 ICP quadrupole mass spectrometer, Thornhill, ON, Canada), using standard addition method with indium as an internal standard. The ICP-MS system was equipped with a GemTip cross-flow nebulizer, Ryton spray chamber, plasma torch with a quartz injector, a Gilson four-channel peristaltic pump (Model Minpuls III) and a Gilson 212B auto-sampler. The assays were done in duplicate.

Two ml of serum was prepared for analyses by diluting 5-times with 0.5% v/v nitric acid and 100 µl of indium solution was added. In the ICP-MS analysis, all the backgrounds of samples and standards determined by reagent blanks were subtracted. The external standard calibration curves (0, 1.0 and 10.0 µg/l of Se) were plotted linearly through zero for isotopes, $^{77}Se^{+}$ and $^{82}Se^{+}$. Hundred and 300 µl of Se standard solution (1000 µg/l) were spiked to 2 serum samples. As $^{77}Se^{+}$ signal is interfered with chloride (i.e. $^{40}Ar^{37}Cl^{+}$), a correction equation was applied for the calculation of Se. ICP-MS instrumental detection limits (IDL) and method detection limits (MDL) for Se analysis in extractable aqueous samples were 0.5 µg/l and 1.0 µg/l, respectively.

The following quality control protocols were implemented for the bovine serum samples analyzed by ICP-MS. The quality of the double distilled water used for preparing reagent blanks, standard solutions, or diluting samples was pre-checked by ICP-MS scanning. National Institute of Standards and Technology (NIST) standard reference material (SRM) Trace Elements in Water (1643d) and SRM Bovine Serum (1598) were analyzed along with the serum samples. The analytical results for Se agreed well with the certified value provided by NIST.

Statistical analyses

Two steers in sequence were exposed to air with SO_2 in experiment 1, therefore, they both were designated as "treated" animals. The results across the exposure periods [pre-exposure (0ppm), 5 ppm, after 5 ppm, 20 ppm and after 20 ppm] are presented as arithmetic means of 2 steers. The steers in experiments 2 and 3 were grouped in blocks involving similar treatments, maintenance, and control conditions since only a few animals could be exposed to SO_2 simultaneously. The research study was performed using a factorial experimental design (treated versus control animals). For the purpose of statistical analysis, we considered animals exposed to 0 and 1 ppm, 0 and 5 ppm, 0 and 20 ppm of SO_2 as separate groups, and carry over effect of the treatments could not be determined. Data were analyzed using Proc GLM (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA) with fixed effect of treatments [SO_2 and control (no SO_2)], environment (warm and cold) where applicable, and two blocks. In experiments 2 and 3, the pre-exposure values for all dependent variables were included as a covariate. In experiment 4 the data were compared with the values that were obtained at exposure steers to 20 ppm of SO_2. Least Square Means (Lsmeans) were estimated using the Pdiff option (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA).

RESULTS

Experiment 1

Vitamin E

Vitamin E concentration in blood plasma of steers was, on average, 7.2 μg/ml during the pre-exposure period (Table 4.1). The level of vitamin E was progressively decreased from the pre-exposure period to 5 ppm, after 5 ppm, 20 ppm and after 20 ppm of SO_2 exposure by 19%, 21%, 29%, and 40%, respectively.

Selenium

Selenium concentration in serum ranged from 64 to 69 ng/g (Table 4.1) and was not affected by exposure to either 5 or 20 ppm levels of SO_2.

Experiment 2

Vitamin E

Comparisons to control animals showed that the mean plasma vitamin E levels in steers were significantly decreased ($P< 0.01$) from 9.12 to 6.12 µg/ml (by 33%) in the steers exposed to 5 ppm level of SO_2 and from 10.98 to 6.04 (by 45%) in steers exposed 20 ppm level of SO_2 (Table 4.2). There was a non-significant ($P> 0.05$) trend for vitamin E to decrease at 1 ppm (9%).

Vitamin A

The concentration of vitamin A in plasma ranged from 2.79 to 3.12 µg/ml in the steers exposed to 1, 5 or 20 ppm levels of SO_2 (Table 4.3) and these values were not significantly different ($P> 0.05$) from the mean plasma level of vitamin A for the control animals, which ranged from 2.23 to 3.08 µg/ml.

Selenium

The mean concentration of Se in serum of steers exposed to 1 ppm of SO_2 was significantly ($p < 0.01$) reduced to 48 ng/g (by 8%) compared to the concentration of 52 ng/g in the corresponding controls. The concentration of Se in animals exposed to 5 and 20 ppm of SO_2 were not significantly different ($P> 0.05$) from the values of Se in serum of control steers (Table 4.4).

Experiment 3

Vitamin E

The concentrations of vitamin E were significantly decreased ($P < 0.05$) in the serum of steers exposed to 5 ppm in the warm environment (by 18%) and to 20 ppm in both warm (by 26%) and cold (by 20%) environments compared to their corresponding controls. There was a non-significant trend ($P > 0.05$) to decrease the level of vitamin E in plasma of steers exposed to 1 ppm (by 13%) and 5 ppm (by 5%) in cold environment (Table 4.5).

Vitamin A

The concentration of vitamin A in control steers ranged from 2.9 to 3.2 µg/ml. The vitamin A concentration in steers maintained in a cold environment was significantly ($P > 0.05$) increased from 3.1 in controls to 3.7 µg/ml (by 19%) during the exposure steers to the 20 ppm level of SO_2 (Table 4.6).

Selenium

Mean Se concentration in bovine serum ranged from 68 to 93 ng/g and was not influenced by either SO_2 exposure or environmental temperature (Table 4.7).

Acute phase proteins

Exposure of steers to the 5 and 20 ppm levels of SO_2 in the warm environment resulted in decreases ($P < 0.05$) in serum levels of orosomucoids from 481 µg/ml in control steers to 412 µg/ml (by 14%). Similar trends were observed in the cold environment, but this effect was not significant ($P > 0.05$). Haptoglobin concentration was <10 µg/ml in all animals and there were no significant changes ($P > 0.05$) with SO_2 exposure in warm or cold environment.

Experiment 4

Vitamins A, E and Selenium

The concentration of vitamin E in plasma and Se in serum of steers supplemented with vitamin E and Se were significantly ($P < 0.05$) higher by 37% and 14%, respectively, compared to control animals. Vitamin A concentration was not affected ($P > 0.05$) by supplementation of vitamin E and Se (Table 4.8).

DISCUSSION

Plasma concentration of Vitamin E decreased in steers exposed to increasing levels of SO_2 in experiments 1, 2 and 3 suggesting that the requirement for vitamin E may have been increased by exposure to SO_2. Although the impact of SO_2 exposure on the actual requirements of vitamin E cannot be predicted from a change in plasma concentration alone, tissue utilization of vitamin E may have increased in order to counteract oxidative stress associated with SO_2 exposure. Further research is required to determine effects of SO_2 exposure on the dietary requirements of vitamin E. The deleterious role of oxygen free radicals and other oxidants in several disease states has been established over the past decade (Grune 2002). Advances in immunology have provided insights into the mechanisms responsible for the effects of various dietary nutrients on specific immune cell functions (Field *et al* 2002). Studies in guinea pigs exposed to 10 ppm of SO_2 support our suggestion that SO_2 inhalation induces oxidant stress in cattle and that oxidative stress increases the demand for vitamin E. As well, the effects of oxidative stress can be reduced by administration of antioxidant vitamins such as vitamin E and C (Etlik *et al* 1995), (Etlik *et al* 1997). In our study vitamin E and Se injection at the end of the SO_2 exposure period significantly ($P < 0.05$) increased blood neutrophil numbers as well as plasma concentration of vitamin E and Se during the subsequent two-week period. This was supported with other studies using human subjects in which bronchial hyperresponsiveness was measured during a 10-minute exposure challenge with SO_2 (0.10 ppm and 0.25 ppm). Subjects who were given dietary antioxidants (400 IU vitamin E and 500 mg vitamin C) responded less severely to the

SO_2 challenge than subjects given a placebo (Trenga *et al* 2001). In another study in Holstein cows, a single injection of 5000 IU of vitamin E followed by oral administration of 3,000 IU of vitamin E per cow per day improved extravasation of neutrophils to the mammary gland at postpartum week 1 and also enhanced neutrophil respiratory responses. The importance of this observation is that rapid recruitment of neutrophils is critical for proper defense against infection and that the immuno-suppression in the cow could be reversed by vitamin E (Politis *et al* 2001).

Retinol (vitamin A) is an essential nutrient that has many physiological effects throughout the body. It may decrease the inflammatory response through inhibition of pulmonary macrophage function, thus resulting in decreased pulmonary injury (Sauer *et al* 1995). In the study being reported, plasma vitamin A at the start of the experiment was within the expected normal range (normal adult bovine serum beta carotene = 300-1,200 µg/dl) (Sherpa International). Vitamin A concentration in plasma increased ($P< 0.05$) after exposure to 20 ppm SO_2, in both the warm and cold environments, but environmental temperature without SO_2 exposure did not influence plasma levels of vitamin A. The reason for the increase in vitamin A in response to SO_2 exposure is not clear but, may have been due to release of vitamin A from tissue storage. Release of vitamin A might be linked to mobilization of lipid from fat depots in association with increases in energy demand and/or stress (Fisher and Placker 1987).

Acute phase proteins (APPs) such as serum amyloid A, haptoglobins (Hpt), and alpha 1-acid glycoproteins (or orosomucoids) have been identified as markers of inflammation in cattle (Alsemgeest *et al* 1994, Horadagoda *et al* 1999). In addition, the circulating concentrations of these proteins in cattle can provide an objective measure of the health status and welfare of an animal. Haptoglobin, for example, has been used to identify both clinical and subclinical disease in animals, and for objectively monitoring the efficacy of antibiotic therapy in experimentally infected animals (Skinner 2001). Level of Hpt is invariably elevated due to increased synthesis during acute and chronic active inflammatory conditions (Skinner 2001). In cattle, the serum concentration of Hpt may increase up to 1000 times within 24 hours of infection, while increases of a lower magnitude indicate the presence of sub-clinical disease (Alsemgeest *et al* 1994, Eckersall 1995). In the present study, the levels of Hpt in the blood serum of the steers exposed to

SO_2 were within the normal range (<10 µg/ml) and did not change. The implications of a decrease in orosomucoids during SO_2 exposure are not clear, since increased values of orosomucoids are usually associated with acute phase inflammatory, neoplastic, degenerative, thrombotic, or traumatic processes (van Dijk *et al* 1995).

Serum Se levels at the start of each experiment were within the expected adequate range for cattle of 0.08 – 0.3 ppm or µg/g (Puls 1994). Selenium concentrations in serum were reduced by 8% during exposure to 1 ppm but not to 5 or 20 ppm SO_2. Since steers were exposed first to 1 ppm, perhaps this represents a transient response to early exposure, which did not persist during subsequent exposure to higher SO_2 levels. This observation may have implications in animals that have a marginal Se status, which is common in Alberta. An epidemiological survey, conducted to measure the Se status in blood of beef cows during the fall season and to determine the risk of Se deficiency among specific geographic regions of Alberta is reported by Campbell *et al* (1995). Soils and plants in Edmonton and Calgary areas were low in Se and 9% of the cows in the study were considered marginal or deficient in Se based on serum concentrations of Se of < 1.27 µmol/l (or < 0.1 ppm) (Campbell *et al* 1995). Selenium, in addition to vitamin E, has antioxidant properties, which serve to protect cellular lipids from oxidative damage *in vivo* (Muller *et al* 2002). It would appear that plasma vitamin E shows marked changes while serum Se remained more stable. Vitamin E and Se interact to compensate to some degree for a deficiency in the other, but it is not clear whether Se can totally compensate for increased vitamin E demands as indicated by decreased plasma levels of vitamin E (Puls 1994).

Collectively, the results of experiments 1, 2, 3 and 4 suggest that close attention to vitamin E and Se status would be advisable in circumstances where cattle are exposed to 5 and 20 ppm of atmospheric SO_2. The increase in blood neutrophil numbers after vitamin E and Se injection suggest a beneficial response. Provision of supplemental dietary vitamin E would likely be beneficial by compensating for the reduced plasma concentration of vitamin E that occurred in steers exposed to SO_2. In these experiments the basal diet was expected to meet the requirements for vitamin E and Se but might potentially fall short in circumstances where dietary vitamin E and/or Se requirements are increased by stress and environmental xenobiotics.

Table 4.1 The arithmetic mean levels of vitamin E in plasma and Se in serum of peripheral blood of 2 steers exposed to 0, 5 and 20 ppm of SO_2. Thermoneutral environment, experiment 1

SO_2 Treatment	Vitamin E (µg/ml)	Selenium (ng/g)
0 ppm	7.2	69
5 ppm	5.8	67
After 5 ppm	5.7	63
20 ppm	5.1	65
After 20 ppm	4.3	64

SEM = standard error
[1] control = atmospheric air

Table 4.2 Level of vitamin E in plasma of control steers and steers exposed to 1, 5 and 20 ppm of SO_2. Thermo-neutral environment, n = 6, experiment 2

	Vitamin E (µg/ml)		
Level of SO_2	[1]Control	SO_2	SEM
1 ppm	7.9	7.2	0.3
5 ppm	9.1[a]	6.1[b]	0.6
20 ppm	11.0[a]	6.0[b]	0.5

ab Means with different superscripts are different ($P< 0.01$)
SEM = standard error
[1] control = atmospheric air

Table 4.3 Level of vitamin A in blood plasma of steers exposed to 0, 1, 5 and 20 ppm of SO_2. Thermo-neutral environment, n = 6, experiment 2

	Vitamin A (µg/ml)		
Level of SO_2	Control	SO_2	SEM
1 ppm	3.1	3.1	0.1
5 ppm	2.2	2.8	0.2
20 ppm	2.6	2.8	0.2

a b Means with different superscripts are different ($P< 0.01$)
SEM = standard error of mean

Table 4.4 Level of selenium in blood serum of control steers and steers exposed to 1, 5 and 20 ppm of SO_2. Thermo-neutral environment, n = 6, experiment 2

	Selenium (ng/g)		
SO_2 level	Control	SO_2	SEM
1 ppm	52[a]	48[b]	1
5 ppm	50	47	2
20 ppm	49	48	1

SEM = standard error of mean

Table 4.5 Vitamin E concentration in plasma of control steers and steers exposed to 1, 5 and 20 ppm SO_2. Effect of warm and cold environments, n = 4, experiment 3

Vitamin E (μg/ml)					
Treatment	Warm		Cold		
Level of SO_2	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	8.2^{ab}	8.7^{a}	7.9^{ab}	6.9^{b}	0.5
5 ppm	10.0^{a}	8.2^{b}	8.2^{b}	7.8^{b}	0.4
20 ppm	9.8^{a}	7.2^{b}	9.9^{a}	7.9^{b}	0.4

a, b = Means within a row with different superscripts are different ($P< 0.05$)
SEM = standard error of the mean
[1] control = atmospheric air

Table 4.6 Concentration of vitamin A in plasma of control steers and steers exposed to 1, 5 and 20 ppm of SO_2. Effect of warm and cold environments, n = 4, experiment 3

	Vitamin A (µg/ml)				
Treatment	Warm		Cold		
Level of SO_2	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	3.2	3.6	3.2	2.9	0.2
5 ppm	2.9	3.0	3.2	3.4	0.2
20 ppm	2.9[b]	3.0[b]	3.1[b]	3.7[a]	0.1

a b Means within a row with different superscripts are different ($P< 0.05$)
[1] control = atmospheric air
SEM = standard error of the mean

Table 4.7 Concentration of selenium in serum of control steers and steers exposed to 1, 5 and 20 ppm of SO_2. Effect of warm and cold environments, n = 4, experiment 3

	Selenium (ng/g)				
Treatment	Warm		Cold		
SO_2 Level	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	68	73	68	73	2
5 ppm	84	76	81	93	5
20 ppm	77	77	81	80	3

[1] control = atmospheric air, 0 ppm
SEM = standard error of mean

Table 4.8 Plasma levels of vitamins E, A and serum level of selenium in steers supplemented with vitamin E and selenium compared to control animals, n = 7, experiment 4

	Treatments		
Parameter	Control	Vitamin E / Se	SEM
Vitamin E (µg/ml)	11.7[b]	18.6[a]	1.8
Vitamin A (µg/ml)	4.6	4.3	0.2
Selenium (ng/g)	50[b]	57[a]	2.0

a, b = the same letters within the row are not significantly different, $P< 0.05$
SEM = pooled standard error of the mean

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Chapter 5

THE EFFECT OF DIFFERENT CONCENTRATIONS OF SULFUR DIOXIDE ON THE PULMONARY CELL COUNT AND NITRIC OXIDE PRODUCTION BY ALVEOLAR MACROPHAGES ISOLATED FROM THE BRONCHO-ALVEOLAR LAVAGE FLUIDS OF CATTLE

INTRODUCTION

Inhalation exposure of the respiratory system occurs when air pollutants and, in particular, sulfur dioxide (SO_2) gas are present in respired air (Koenig *et al* 1983). The upper and lower airways of the respiratory tract are covered by a continuous layer of epithelium on which is a protective mucous layer. The airway-coating layer, which is 95-98% water rich in electrolytes and proteins, is a neutralizer for many toxins and an important component of the mucociliary apparatus (Hulbert *et al* 1989). Alveolar macrophages have been identified as distinct functional and morphological entities providing protective phagocytic function in the lung (Bowden 1976). The phagocytic activity of macrophages, in combination with the mucociliary clearance mechanism, helps to protect the lungs from intrinsic cell debris, inhaled dust, and microorganisms. However, inhaled atmospheric pollutants may affect macrophage function leading to lung disease (Bowden 1971). Nitric oxide (NO), a free radical, is an important component in the microbial killing function of macrophages. It has cytotoxic effects on a number of microbes, parasites, and tumor cells (Bochsler *et al* 1996).

The host defense mechanism against inhaled pathogens and foreign matter includes mucociliary and pulmonary clearance. Sulfuric acid (H_2SO_4), which is formed as a result of SO_2 contacting moist mucous membranes, depresses pulmonary particle clearance (Amdur 1989). Degradation of the pulmonary surfactant resulting in a slowdown of the pulmonary clearance rate and an increase in the lung particulate burden suggests the possibility of adverse health effects in the case of chronic inhalation of SO_2 even at low concentrations or after short exposures to strongly contaminated air (Podgorski *et al* 2001). Therefore, a healthy pulmonary system is required to protect the animal from the deleterious effects of air pollution. Besides the pulmonary clearance mechanisms, the respiratory system also possesses the phagocytic defense of alveolar macrophages. Macrophages are not only concerned with phagocytosis of infective agents

or other antigenic components, but they also present antigens to the immune cells in such a manner as to initiate an immune response (Aderem and Underhill 1999). Macrophages are essential to the healing of wounds and repair of tissues damaged by inflammation (Tsunawaki *et al* 1988), and play a central role in host defense in the lower respiratory tract. Production of the reactive intermediate NO, via expression of inducible nitric oxide synthase, is an important microbicidal effector mechanism possessed by macrophages (Mason *et al* 1996). Nitric oxide exerts cytotoxic actions on microbes, parasites, and tumor cells (Bochsler *et al* 1996). The antimicrobial and cytotoxic actions of NO are enhanced by other macrophage products such as hydrogen peroxide, or superoxide (MacMicking *et al* 1997).

In this study we tested the hypothesis that the health status of the pulmonary cells and defense activity of pulmonary macrophages (level of nitric oxide production) are altered in cattle exposed to atmospheric SO_2. A second hypothesis is that cold weather has interactive effects with atmospheric SO_2 to modify pulmonary defenses. The objective of the study was to identify concentrations and cumulative exposure effects of SO_2 in cattle and to test the above hypotheses concerning pulmonary cells and defenses.

MATERIALS AND METHODS

Two experiments with growing Simmental steers were performed. The details on project and housing, feeding and maintaining of the animals are given in Chapter 3.

***Experiment 2** (exposure to 0, 1, 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Twelve steers 6 to 9 months of age, and with an initial body weight averaging 391 kg, were used in this experiment that was performed in a thermo-neutral environment (+17 to +20 °C). The animals were fed a ration at a level calculated to provide approximately 2.2 times maintenance requirements (NRC 1996). A group of six steers was sequentially exposed to atmospheres with 1 ppm for 23 days, 5 ppm for 9 days, and 20 ppm of SO_2 for 8 days. A similar group of 6 control steers was exposed to ambient air ("0 ppm of SO_2") in hoods during the designated experimental exposure periods.

***Experiment 3** (exposure to 0, 1, 5, and 20 ppm of SO_2 in thermo-neutral and cold environments)*

Sixteen steers, 12 to 14 months of age, and with an initial body weight averaging 534 kg, were used in this experiment. These animals were fed a ration at a level calculated to provide approximately 2.2 times maintenance energy requirement. A group of 8 steers was sequentially exposed to atmospheres amended with 1 ppm for 10 days, and then 5 and 20 ppm of SO_2 for 7 days at each level. This experiment was carried out simultaneously in two different thermal environments: thermo-neutral (+17 to +20 °C) and cold (-15 to -17°C) designated as "warm" and "cold" respectively. The steers and hoods were in a temperature controlled chamber. These animals, unlike in previous 2 experiments, were subjected to 2 different temperature treatments. A similar group of 8 control steers was exposed to ambient air in each temperature environment ("0 ppm of SO_2") during the designated exposure periods.

Duration of the SO_2 exposure

In all 3 experiments, the animals were exposed to atmospheres with SO_2 or ambient air for 6 hours/ day for 5 consecutive days. Within experiments 2 and 3, the steers were randomly allocated to experimental and control groups.

Initial Values of Tested Parameters

A pre-exposure period of 14 days in duration was conducted at the beginning of each experiment in order to obtain the animals' initial values for the tested parameters, as well as to evaluate the initial health status of the steers. During this period [study days (-14) to (0)], in which a thermo-neutral and cold environments (where applicable) were maintained, all planned test procedures were performed.

Sample Collection

Bronchoalveolar lavage fluid (BAL) procedure

Broncho-alveolar lavage specimens were collected during the pre-exposure period and once on the day after the end of each SO_2 exposure period. The BAL specimen was collected using a sterile Bivona broncho-alveolar lavage catheter with inflatable cuff (CDMV2567, CDMV, Ontario, Canada). Briefly, the nasal membranes were desensitized with topical lidocaine, and the BAL catheter, lubricated with a sterile water soluble lubricant (K-Y Jelly) was inserted through the nasal pharynx into the trachea and the insertion was stopped when moderate resistance to movement of the BAL catheter was determined. The cuff was inflated and sterile saline (180 ml in 3 aliquots) was administered into a broncho-alveolar area and subsequently withdrawn. The cuff was deflated and the BAL catheter removed.

Necessary equipment: sterilized 2 meter long Bivona catheters, 3-way stopcocks, warm (37°C) sterile pyrogen free saline (Baxter Healthcare Corporation, Deerfield, IL) 60cc, 20cc, and 6cc syringes purchased from (Fisher Sci, Edmonton, AB, Canada), 2% lidocaine (MTC Pharmaceuticals, USA), 50 ml sterile conical tubes with caps (Falcon, 2070, Becton Dickinson Labware, New Jersey, USA), K-Y Jelly (Medical Supply Company, Inc., Bethpage, NY, USA), and a bucket with ice.

Isolation of pulmonary cells from BAL

Alveolar macrophages (AM) and other pulmonary cells such as lymphocytes, neutrophils, epithelial cells, including degenerative (damage) cells were isolated from the BAL fluid by modifications of previously described procedure by Stanley *et al* (1991). The BAL fluid was filtered through two layers of sterile gauze into 50-mL polypropylene tubes and centrifuged at 2000 rpm for 15 min at 4°C. The recovered supernatant was collected and stored at -80°C for lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) assays. Cells from the same animal were then pooled together and washed with chilled sterile phosphate saline buffer (1xPBS) with subsequent centrifugation at 2000 ppm for 7 min. The pellet was resuspended in 15 ml of PBS containing 1% v/v fetal bovine serum (FBS), (Gibco Invitrogen Corporation, Burlington, ON), and 20 µl of the

cell suspension from each sample was taken to determine total cell numbers. The culture tubes were centrifuged at 2000 rpm for 5 min. The obtained pellet was resuspended in 1.8 ml of complete culture media (CCM) composed of sterile RPMI 1640 (Sigma Chemicals CO, R-8758, Levis, MO, USA) supplemented with 4% v/v sterile FCS, 1% v/v Antibiotic/antimycotic mixture (catalog 15240-062, Gibco Invitrogen Corporation, Burlington, ON) and sterile 0.25% v/v of 1M Hepes buffer solution (catalog 15630-080, Gibco Invitrogen Corporation, Burlington, ON). This cell suspension was used for the nitric oxide (NO) assay.

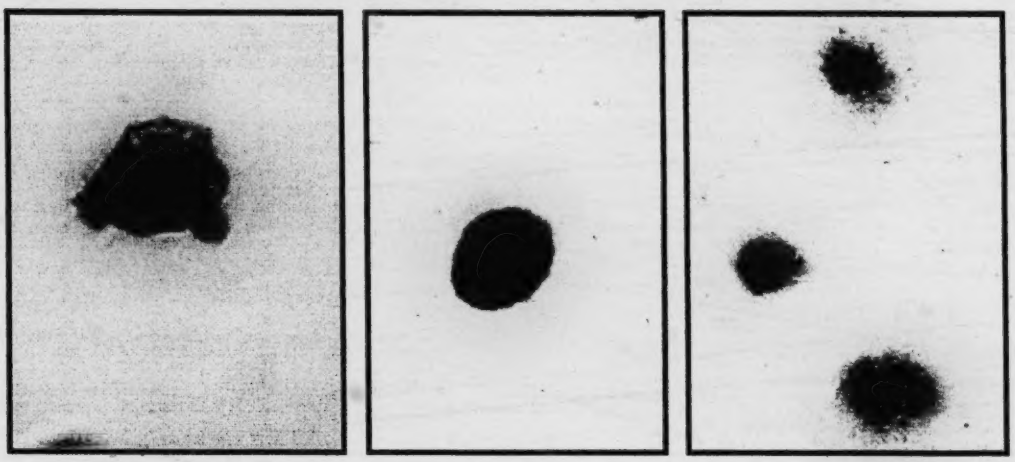
Total cell count

A hemocytometer technique was used to determine the total number of cells, and cellular viability was determined using the Trypan-blue exclusion test. All specimens had greater than 98% viability.

Differential cell count

The cells were placed on glass slides for morphologic evaluation using techniques described by Coles (1986) for blood smears. The prepared slides were air dried for 5 min, and immediately fixed with a cytology fixative (CytoPrep, 12-570-10, Fisher Scientific, Nepean, Ontario, Canada). They were stained by the Gimza-Romanovsky method using an automated stainer. The cell differential count on each slide was determined evaluating 200 cells morphologically, and each slide was evaluated 2 times. Morphological evaluation was identification of normal appearing alveolar macrophages, epithelial cells, lymphocytes, neutrophils and non-identified degenerative cells and inclusion or exclusion of the vital stain. Non-degenerative cells had clearly identified or typical nuclei for each cell type, smooth cell membranes with characteristic staining and morphology of the cytoplasm. Degenerative cells had pycnotic or fragmented nuclei, and the staining characteristics and morphology was not characteristic of a normal cell. Degenerative cells often had damaged cell membrane with "bleeding" cytoplasm (Figure 5.1). The cell viability data ratio was determined by total degenerative/ total normal cells ratio.

FIGURE 5.1 Photographic images of representative normal and degenerative cells isolated from the BAL specimens. Magnification 40 x.



Macrophage Alveolar cell Degenerative cells

Preparation of cells for a nitric oxide assay

Cell suspension (200 µl) from each specimen was added into 8 wells of a sterile 96-well flat bottom micro-titer plate (Fisher Scientific, Edmonton, AB, Canada). To allow the microphages to adhere to the bottom of the wells, the prepared plate was incubated for 2 h in a humidified atmosphere at 37°C in the presence of 5 % CO_2 . After incubation, non-adherent cells were removed by washing the wells 3 times with 200 µl of sterile Krebs' Ringer HEPES buffer (pH 7.4) at room temperature. Half of the cultured wells were challenged with lipopolysaccharide mitogen (LPS) purchased from Sigma Chemicals, St. Louis, MO, (1µg/ml), the another half served as a control. Macrophages were incubated for an additional 24 hours in the previously described conditions. After incubation, the culture supernatant from each well was transferred to 0.6-µl microcentrifuge tubes and stored at -80°C for the nitric oxide (NO) immunoassay.

Nitric oxide colorimetric assay

Nitric oxide production in culture supernatants collected from unstimulated and LPS-stimulated alveolar macrophages was determined by analyzing for nitrite (NO_2^{1-}) concentration using a colorimetric assay based on the Griess reaction (Green et al 1982). All Griess reagent chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA). The assay was performed in non-sterile 96-well flat bottom microtiter plates (Fisher Scientific, Edmonton, AB, Canada). Macrophage culture supernatant (50 µl) or sodium nitrite standard (50 µl) was combined with 50 µl of Griess reagent prepared from 1 volume of 0.5% w/v sulfanilamide in 6% v/v phosphoric acid with 1 volume of 0.05% w/v naphthylethylene-diamine dihydrochloride in distilled water in corresponding wells. All standards and samples were done in quadruplicate. The absorbance was measured at 540 nm in an automatic UV *max* kinetic microplate reader (Molecular Device, USA) and NO_2^{1-} concentrations were determined with reference to a standard curve generated with sodium nitrite (10 to 100 µg/ml) in complete culture media. The values were subsequently converted into the amount of nitrite generated on a cell basis. The data were expressed as µg of nitric oxide per 10,000 of alveolar macrophages.

Indirect immunofluorescence leukocyte (phenotype) assay

The flow cytometry immunoassay with the use of CD14 (cell line CAM36A) monoclonal antibody (Mab) was performed to determine that the given sample has a detectable population of macrophages, and with Mab CD3 (cell line MM1A) to verify the subpopulation of lymphocytes. The Mab were purchased from VMRD, Inc. Pullman, WA 99163 USA.

The assay was performed in non-sterile V-bottom 96-well micro-titer plates (Fisher Scientific, Edmonton, AB, Canada). The alveolar cell culture was freed from CCM by centrifugation at 2000 ppm for 30 seconds and resuspended in 200 µl of cold wash buffer (1x PBS plus 1% of FBS). Fifty µl of the cells were added into 4 wells marked as background (Bgrd), FITC, CD3, and CD14. Fifty µl of diluted (1:100) monoclonal antibodies (Mab) were added to the corresponding CD3 and CD14 wells, and 50 µl of wash buffer was added into "Bgrd" and "FITC" wells. The plate was incubated for 30 minutes on ice in the dark. After the incubation the wells were washed 3 times

with cold wash buffer and centrifuged at 2000 rpm for 1 minute. Wash buffer (50 µl) was added into the "Bgrd" well and 50 µl of diluted FITC (1:100) was added into the remainder of the wells. The plate was incubated again for 30 minutes on ice in the dark. The cell pellets were washed 2 times and resuspended in 200 µl of 1x PBS plus 2% formaldehyde. The labeled cells were stored at 4°C until the next day and were acquired on FACScan. In order to identify the cell population of interest based on immunofluorescence, a gate (a light scattering window) is set to include the majority of the lymphocytes and another gate is selected for the monocytes. In this manner, maximal recovery of the lymphocytes and non-lymphocytes within a sample can be consistently obtained (Loken et al 1990).

Lactate dehydrogenase (LDH) and *Alkaline Phosphatase (ALP)*

The activities of LDH and ALP in BAL fluids were determined on an automated multiple-channel analyzer Hitachi 704 (Hitachi, Roche Diagnostics, Laval, Quebec), according to manufacture's procedures.

Statistical Analyses

The steers in experiments 2 and 3 were grouped in blocks involving similar treatments, maintenance, and control conditions. The research study was performed using a factorial experimental design (treated versus control animals). For the purpose of statistical analysis, we considered animals exposed to 0 and 1 ppm, 0 and 5 ppm, 0 and 20 ppm of SO_2 as separate groups and carry over effect of the treatments could not be determined. Data were analyzed using Proc GLM with fixed effect of treatments [SO_2 exposed and control (no exposure to SO_2)], environment (warm and cold) where applicable, and 2 blocks. In experiments 2 and 3, the pre-exposure values for all dependent variables were included as a covariate. In case of nitric oxide production the no-LPS values were compared with the LPS values separately in control and in exposed to SO_2 steers due to small degree of freedom. Least Square Means (Lsmeans) were estimated using the Pdiff option (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA).

RESULTS

Experiment 2

Pulmonary cells

Differential cell counts included non-degenerative or normal lymphocytes, neutrophils, macrophages and pulmonary epithelial cells and degenerative cells.

The ratio of degenerative to normal pulmonary cells in BAL specimens in each treatment is summarized (Table 5.1). There was a significant ($P< 0.05$) 3-fold increase in the ratio of degenerative to normal cells at 20 ppm level of SO_2 compared to control treatment. Similar (about 4-fold) but non-significant ($P> 0.05$) trends to increase were observed at both 1 and 5 ppm levels of SO_2 exposure.

Pulmonary epithelial cells The percentage of pulmonary epithelial cells was significantly ($P< 0.001$) increased by 29% in steers exposed to 20 ppm of SO_2 compared to their control steers. There were no changes after exposure steers to 1 ppm ($P= 0.7$) or to 5 ppm ($P= 0.1$) of SO_2 (Table 5.2).

Pulmonary macrophages The percentages of pulmonary macrophages were significantly ($P< 0.001$) decreased by 8, 16 and 52% in steers exposed to 1, 5 and 20 ppm of SO_2 respectively, compared to their control steers (Table 5.2).

Pulmonary neutrophils The percentages of pulmonary neutrophils were significantly ($P< 0.001$) increased by 2.5, 3.5 and 5.4-fold in steers exposed to 1, 5 and 20 ppm of SO_2 respectively, compared to their control steers (Table 5.2).

Pulmonary lymphocytes The percentages of pulmonary lymphocytes were not different ($P= 0.24$) in steers exposed to 1, 5 and 20 ppm of SO_2 compared to their control steers (Table 5.2)

Nitric Oxide (NO) production by pulmonary macrophages

Basal level of NO (Figure 5.2). The basal rate of NO oxide production ranged between 6.5 and 7.1 $\mu g/10^{-4}$ cells. There was no difference ($P= 0.9$) in the production of NO by non-stimulated pulmonary macrophages between control group of steers and steers exposed to 1, 5 and 20 ppm of SO_2.

NO production after stimulation of macrophages with LPS (Figure 5.3). The ratio of NO production by macrophages stimulated with LPS to the non-stimulated macrophages was 2-fold lower in steers exposed to 20 ppm of SO_2 (P= 0.001) compared to their corresponding controls. There was a slight trend (from 4.6 to 4.1) to decrease NO production in steers exposed to 1 ppm of SO_2 (P= 0.2). There was larger but also not significant (P= 0.06) decrease in ratio (from 4.9 to 3.6) in steers exposed to 20 ppm of SO_2 compared to their corresponding controls. In this experiment only 4 steers were used. If there were 8 steers in this experiment the difference between 4.9 and 3.6 (P< 0.06) would be significant at P< 0.05 (calculations are presented on the page 135).

Lactate dehydrogenase (LDH) activity in BAL fluids

The activity of LDH in the BAL specimens collected from steers exposed to 1 and 20 ppm levels of SO_2 was significantly higher (2-fold, p< 0.05) than the values determined in the BAL specimens collected from their corresponding control animals (Figure 5.4). There was also a trend towards an increase in LDH activity in response to exposure at the 5 ppm SO_2 level (P = 0.09).

Alkaline Phosphatase (ALP) activity in BAL fluids

The concentration of ALP in BAL specimens is expressed as a change from the pre-exposure value, which was relatively constant during experimental periods in control animals. The mean ALP activity for the animals exposed to 20 ppm level was 30-fold higher (P< 0.05) then the ALP activity of BAL samples from their corresponding control animals. The values of ALP in BAL of steers exposed to 1 and 5 ppm levels of SO_2 were not significantly (P> 0.05) different from controls (Figure 5.5).

Experiment 3

Pulmonary cells

The ratios of degenerative to normal pulmonary cells in BAL fluids are summarized by treatment group (Table 5.1). There was a 2.2-fold increase ($P< 0.05$) in the ratio of degenerative to normal cells at 20 ppm level of SO_2 exposure compared to controls in both the warm and cold environments. Similar trends were also present at both 1 and 5 ppm SO_2 exposure levels, but these trends were not statistically significant ($P> 0.05$).

Pulmonary epithelial cells The percentages of pulmonary epithelial cells were significantly increased by 77% ($P< 0.001$) and 55% ($P= 0.03$) after exposure steers to 5 and 20 ppm of SO_2 respectively, compared to their controls in the warm environment. In the cold environment there were significant increases in the percentage of pulmonary epithelial cells after exposure steers to 1 ppm by 32% ($P= 0.004$) and 20 ppm by 32% ($P= 0.005$). There was a trend ($P=0.06$) to increase the percentage of epithelial cells by 17% after exposure of steers to 5 ppm of SO_2 (Table 5.4).

Pulmonary macrophages The percentages of pulmonary macrophages were significantly ($P< 0.001$) decreased by 20%, 40% and 40% in the warm environment and by 20%, 20% and 30% in the cold environment in steers exposed to 1, 5 and 20 ppm of SO_2 respectively, compared to controls. There was a significant ($P= 0.004$) interaction of environmental temperature and SO_2 treatments (Table 5.4).

Pulmonary neutrophils The percentages of pulmonary neutrophils were significantly ($P< 0.001$) increased by 0.9, 4 and 2.3-fold in the warm environment and by 1.8, 2.4 and 4.4-fold in the cold environment in steers exposed to 1, 5 and 20 ppm of SO_2 respectively, compared to their controls (Table 5.4).

Pulmonary lymphocytes The percentages of pulmonary lymphocytes were not different ($P= 0.2454$) in steers exposed to 1, 5 and 20 ppm of SO_2 compared to the control steers in both warm and cold environments (Table 5.4).

Nitric Oxide (NO) production by alveolar macrophages

Basal level of NO (warm environment) (Figure 5.6). The basal level of NO oxide ranged between 5.7 and 7.4 $\mu g/10^{-4}$ cells. There was no difference ($P= 0.4$) in the

production of NO by non-stimulated pulmonary macrophages between control group of steers and steers exposed to 1, 5 and 20 ppm of SO_2.

Basal level of NO (cold environment) (Figure 5.7). The basal level of NO oxide ranged between 4.8 and 7.2 $\mu g/10^{-4}$ cells. There was no difference (P= 0.4) in the production of NO by non-stimulated pulmonary macrophages between control steers and steers exposed to 1, 5 and 20 ppm of SO_2.

NO production in macrophages stimulated with LPS (warm environment) (Figure 5.8). The ratio of NO production by stimulated with LPS macrophages to the non-stimulated macrophages in the thermo-neutral environment was 130% (P=0.04), 180% (P< 0.001) and 240% (P< 0.001) lower in steers exposed to 1, 5 and 20 ppm of SO_2, respectively, compared to the corresponding control steers.

NO production in macrophages stimulated with LPS (cold environment) (Figure 5.9). The ratio of NO production by macrophages stimulated with LPS to the non-stimulated macrophages in the cold environment was 160% lower (P= 0.001) in steers exposed to 20 ppm of SO_2 compared to their corresponding control steers. There was no difference (P> 0.05) in the production of NO by stimulated macrophages between control steers and steers exposed to 1 and 5 ppm of SO_2.

Lactate dehydrogenase (LDH)

There was no effect of the thermal environment on the values for LDH activity. Since thermal environment did not have an effect on LDH activity, the values for warm and cold environments were pooled (Figure 5.10). The activity of LDH in the BAL samples isolated from the animals exposed to 20 ppm was increased (P< 0.05) by 4-fold compared to the control animals. The activity of LDH for the steers exposed to 1 and 5 ppm levels of SO_2 were not significantly (P> 0.05) different from control steers. However, there was a trend of 2-fold increased activity of LDH at 5 ppm of SO_2 exposure.

Alkaline Phosphatase (ALP)

As in experiment 2, the ALP activity values varied markedly among animals and data are therefore presented as changes from pre-exposure values. In this experiment

there was no effect of the thermal environment on ALP activity, therefore, the data obtained from the animals housed in warm and cold environments were pooled and presented as LSmeans ± SEM. The ALP values were 100-fold higher ($P< 0.05$) in the BAL samples from the animals exposed to 20 ppm level of SO_2 compared to the corresponding controls. The values of ALP obtained for steers exposed to 1 and 5 ppm were not significantly ($P> 0.05$) different from values for the control steers (Figure 5.11). However, there was a trend of about 50-fold to increase the activity of ALP at these SO_2 concentrations.

DISCUSSION

Analysis of bronchoalveolar lavage fluid is an effective method of detecting an inflammatory response in the lungs of animals in toxicological studies. The increased ratio of degenerative to normal pulmonary cells including increased percentage of epithelial pulmonary cells, after exposure to 20 ppm of SO_2 in warm and cold environments indicates pulmonary damage. Sulfur dioxide-induced epithelial cell injuries have been reported in rabbits exposed to 20 ppm of SO_2 for 4 hours per day for 4 weeks (Sugiura *et al* 1997). Sulfur dioxide gas is a strong oxidant that can react with many biochemical moieties to form free radicals (Hippeli et al 1997). Free radicals formed by this interaction adversely affect the structure and function of cellular components such as proteins, nucleic acids, and the cellular plasma membrane. The decreased production of NO by alveolar macrophages challenged with LPS suggests a reduced microbial killing ability of the macrophages since highly reactive nitric oxide is generated in order to kill pathogens (Bochsler et al 1996). In rats exposed to SO_2, inflammatory cells were found in BAL fluid in numbers significantly higher from those observed in healthy controls (Krasnowska *et al* 1998). Exposure to SO_2 or sulfite aerosols induce inflammatory reactions in the respiratory tract characterized by an influx of neutrophils into the airways (Beck-Speier *et al* 1994), which is a common feature observed during pulmonary inflammation induced by air pollutants, including SO_2 and sulfates (Labbe *et al* 1998). In our study we also observed increased percentage of neutrophils in steers exposed to all three levels of SO_2 in warm and cold environments compared to the control steers.

We also report decreased percentage of pulmonary macrophages after exposure of steers to 1, 5 and 20 ppm of SO_2 compared to the control steers. An *in vitro* study (Knorst *et al* 1996) showed a dose-dependent reduction in the migration rate of blood monocytes and alveolar macrophages exposed to 0.5, 1.5 and 2.5 ppm of SO_2. The authors suggested that the decrease in cell migration induced by SO_2 was due to changes in chemotactic mechanisms and not to cell death. In the study being reported, it is possible that breathing the air with SO_2 did not affect the migration rate of the blood monocytes and allowed them to leave blood vessels at the pulmonary sites. However, in lung tissues, where macrophages underwent the direct exposure to SO_2, their numbers were decreased and as well, the cytotoxic function was affected, resulting in the decreased production of NO at all levels of SO_2 exposure in a thermoneutral environment. Nitric oxide is synthesized from L-arginine by the family of NO synthases (NOS). Nitric oxide synthase 2 (NOS2) is an inducible membrane-bound enzyme responsible for production of NO in monocytes and macrophages during infection or inflammation (Moilanen *et al* 1997). Many cellular components are involved in a very complex mechanism of biosynthesis and functioning of NOS2 (Kuncewicz *et al* 2001). It is possible that SO_2 interacts with this mechanism by causing damage to the cell membrane due to lipid peroxidation process (Kucukatay et al 2003) or interferes with the other components. This will require further investigation on how SO_2 affects production of NO by alveolar macrophages.

Elevation of LDH in the BAL fluid is an indicator of cytotoxicity. Exposure of rats to SO_2 increased the activity of LDH in BAL lavage fluid (Krasnowska et al 1998). In this study being reported, we observed an increase in the activity of LDH in BAL fluid from cattle exposed to 20 ppm level of SO_2. Since LDH is a cytoplasmic enzyme that is released from cells after damage to the cell membrane, an increase in LDH activity was also consistent with the increased ratio of degenerative to healthy pulmonary cells isolated from the BAL fluid of steers exposed to SO_2. Examination of alterations in BAL in rats and mice during chronic exposure to high levels of diluted diesel exhaust revealed increasing levels of LDH in BAL correlated with the development of pulmonary fibrosis (Henderson et al 1985). Enzymatic activities of the alveolar macrophages in BAL fluid from animals injected with SO_2-sorbed chrysotile also showed a significant increase of the enzyme LDH (Jaurand *et al* 1978).

The increases in ALP activity observed in response to increasing exposure levels of SO_2 were also likely a reflection of cellular pulmonary damage (Henderson et al 1987). ALP together with LDH in BAL fluids are useful indicators of cell damage in the pulmonary tract of cattle. Pulmonary cell damage in cattle caused by SO_2 may influence the ability of the animal to achieve effective bronchial clearance, as observed in donkeys (Spiegelmann et al 1968). These observations have important health implications for cattle challenged with respiratory pathogens.

The results of experiment 3 indicate that environmental temperature did not appear to exert any direct effect on integrity of pulmonary cells as indicated by LDH and ALP activities in BAL fluid and ratio of degenerative to normal cells. However, cold stress decreased the production of NO by alveolar macrophages in control animals and in the animals exposed to 20 ppm of SO_2 (SO_2 and environment interaction) ($P< 0.09$). The mechanism by which cold exposure suppressed NO production by macrophages is not known and requires further investigation. Results of the two experiments in this study support the hypothesis that exposure to atmospheres with SO_2 causes injury to pulmonary system of cattle resulting in increased ratio of degenerative to normal pulmonary cells and suppressed production of NO by pulmonary macrophages. In addition, the decreased percentage of pulmonary macrophages and increased percentage of neutrophils in lungs in response to exposure to all experimental levels of SO_2 in experiment 3 were in general agreement with observations in experiment 2. These assessments of cells in BAL fluid indicated damage in the lung tissue as a result of exposure of steers to atmospheric SO_2.

Table 5.1 Ratio of degenerative to normal pulmonary cells[1] (n = 6) in smears prepared from BAL fluids from control steers and steers exposed to 1, 5 and 20 ppm levels of SO_2, experiment 2.

	Treatment		
Level of SO_2	Control[2]	SO_2	SEM
1 ppm	0.3	1.3	0.4
5 ppm	0.4	1.8	0.5
20 ppm	0.5^b	1.5^a	0.2

a b means within a row with different superscripts are different ($P < 0.05$).

[1] Differential counts included lymphocytes, neutrophils, macrophages, epithelial cells and degenerative cells.

[2] control = atmospheric air

SEM – standard error of the mean

Table 5.2 Differential cell count performed on the cells isolated from bronchoalveolar lavage fluids obtained from control steers and steers exposed to 1, 5 and 20 ppm of SO_2. Data presented as percentage (Lsmeans) of the total cell count, n = 6, experiment 2

Type of the cells	Level of SO_2	Treatment		SEM
		Control[1]	SO_2	
Epithelial cells	1 ppm	47.3	48	1.52
	5 ppm	47.0	50.7	1.52
	20 ppm	47.5^b	62.1^a	1.52
Macrophages	1 ppm	50.3^a	46.4^b	1.32
	5 ppm	50.4^a	42.0^b	1.32
	20 ppm	49.8^a	26.1^b	1.32
Neutrophils	1 ppm	1.8^a	4.5^b	0.51
	5 ppm	1.9^a	6.7^b	0.51
	20 ppm	1.9^a	10.3^b	0.51
Lymphocytes	1 ppm	0.5	0.9	0.27
	5 ppm	0.6	0.6	0.27
	20 ppm	0.8	1.5	0.27

a b – Different letters denote significant differences between control and exposed to 1, 5 and 20 ppm of SO_2 steers, $P< 0.05$
SEM - standard error of the mean
[1] control - atmospheric air

Figure 5.2 Nitric oxide production by alveolar macrophages (basal level of NO) that were not stimulated with LPS, n = 6, experiment 2

Bars comparing NO production by non-stimulated macrophages within and between 1, 5, and 20 ppm of SO_2.

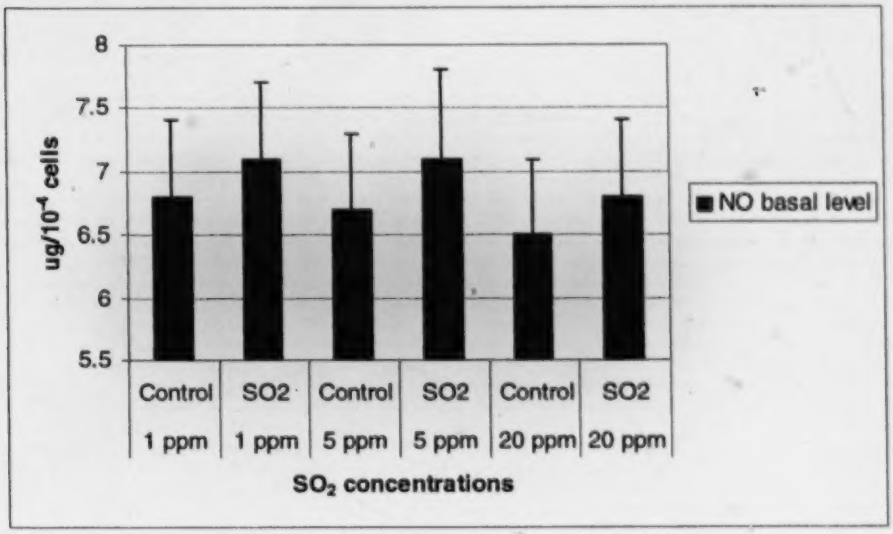
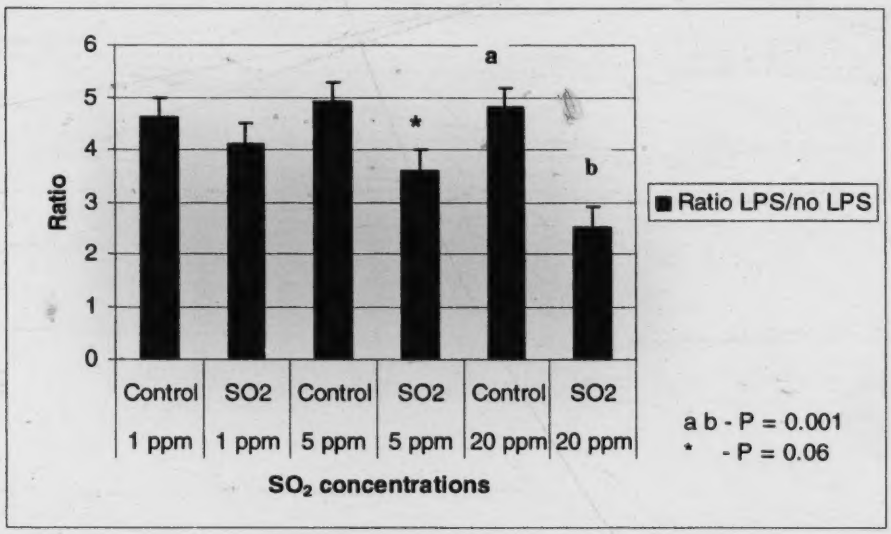


Figure 5.3 Ratio of NO production by pulmonary macrophages stimulated to non-stimulated with LPS isolated from BAL of control steers and steers exposed to 1, 5 and 20 ppm of SO_2, n = 6, experiment 2.

Bars comparing the ratio of NO production by stimulated with LPS to non-stimulated macrophages within one level of SO_2 only.



a b – Different letters denote significant differences between control and exposed to 1, 5 and 20 ppm of SO_2 steers, $P< 0.05$

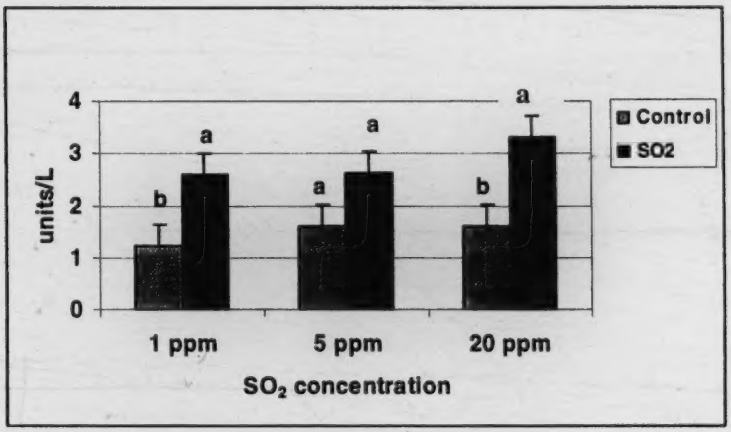
* - P = 0.06 at exposure steers to 5 ppm compared to controls

*Sample size calculations (P= 0.06): the procedure by Stein (Steel *et al* 1997)

$N = (t^2 * s^2)/ d^2$,
where N is a sample size, $t = 1.812$ is a tabulated value for confidence level of 95% and degree of freedom (df) is $n_1 + n_2 - 2) = 10$; standard deviation (SD) = $SE*\sqrt{6} = 0.4*2.45 = 0.98$, d is half width of the confidence interval that is $\mu_1 - \mu_2 = 4.9 - 3.6 = 1.3/2 = 0.65$.

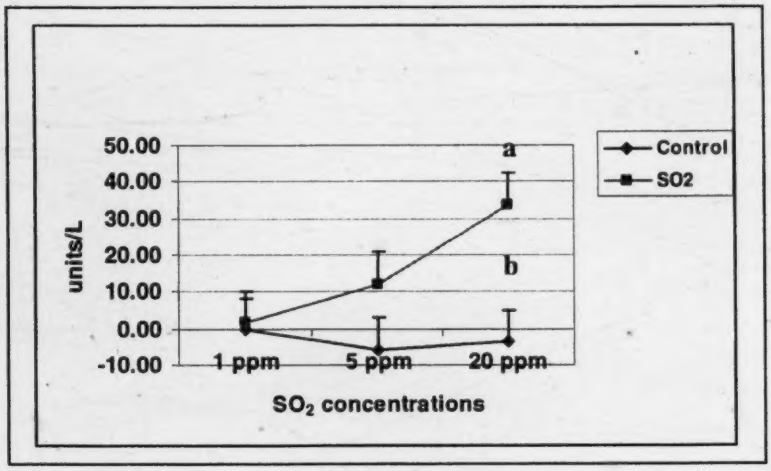
$N = [(1.812)^2 * 0.98^2]/(0.65)^2 = 3.32*0.98/0.4 = 8$
Sample size of 8 steers would make this difference significant at $P< 0.05$.
Power of the test is 60%.

Figure 5.4 Activity of lactate dehydrogenase (LDH) in BAL fluids collected from the steers exposed to 0, 1, 5, 20 ppm levels of SO_2, n = 6, experiment 2



a, b – Different letters denote significant differences between control group and groups exposed to SO_2, $P < 0.05$

Figure 5.5 Activity of ALP in BAL specimens collected from control steers and the steers exposed to 1, 5, 20 ppm levels of SO_2. The activity of ALP in BAL specimens is expressed as a change from the pre-exposure value, n = 6, experiment 2



a, b – comparison between control and exposed to SO_2 animals within the same exposure level, $P< 0.05$

Table 5.3 Ratio of degenerative to normal pulmonary cells in smears prepared from BAL fluids collected from control steers and steers exposed to 1, 5, and 20 ppm levels of SO_2 in warm and cold environments, n = 4, experiment 3.

	Treatment				
	Warm		Cold		
Level of SO_2	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	0.3	0.6	0.3	0.4	0.1
5 ppm	0.4	0.8	0.3	0.5	0.1
20 ppm	0.4^{b}	0.9^{a}	0.3^{b}	0.7^{a}	0.1

a b, means within a row with different superscripts differ ($P < 0.05$).
SEM = standard error of the mean;
[1] control = atmospheric air.

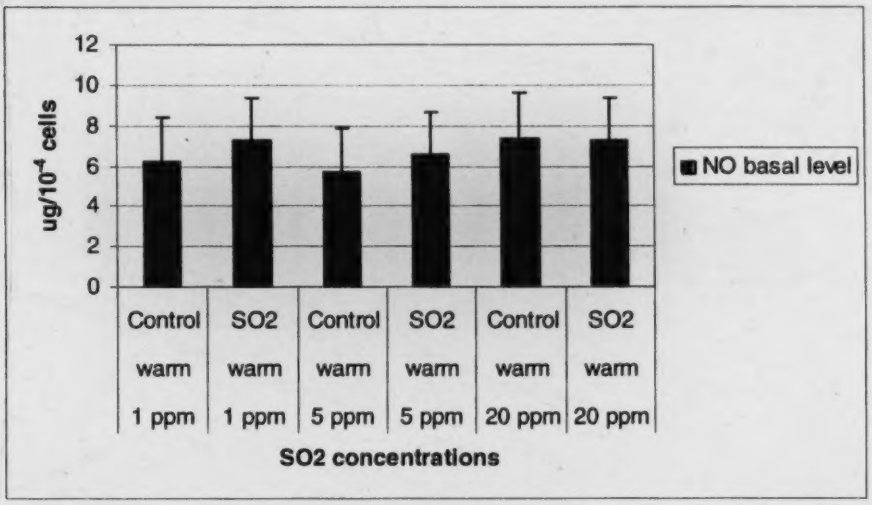
Table 5.4 Differential cell count performed on the cells isolated from BAL fluids obtained from control steers and steers exposed to 1, 5 and 20 ppm of SO_2 housed in warm and cold environments. Data presented as percentage (Lsmeans) of the total cell count, n = 6, experiment 3

		Treatment				
Type of the cells	Level of SO_2	Warm		Cold		
		Control[1]	SO_2	Control[1]	SO_2	SEM
Epithelial cells	1 ppm	35.4	42.1	34.1^{b}	45.0^{a}	1.52
	5 ppm	30.0^{b}	53.2^{a}	40.1	47.1^{*}	1.52
	20 ppm	33.5^{b}	51.9^{a}	33.7^{b}	44.4^{a}	1.52
Macrophages	1 ppm	60.9^{a}	51.6^{b}	62.5^{a}	49.0^{b}	2.29
	5 ppm	66.9^{a}	37.6^{b}	56.0^{a}	44.5^{b}	2.29
	20 ppm	62.9^{a}	35.1^{b}	62.8^{a}	43.8^{b}	2.29
Neutrophils	1 ppm	2.3^{b}	4.4^{a}	1.6^{bc}	4.4^{a}	0.55
	5 ppm	2.0^{b}	11.7^{a}	2.0^{b}	6.7^{a}	0.55
	20 ppm	2.3^{b}	7.7^{a}	2.0^{b}	10.8^{a}	0.55
Lymphocytes	1 ppm	1.4	1.8	1.9	1.6	0.28
	5 ppm	1.1	1.4	2.0	1.7	0.28
	20 ppm	1.2	1.3	1.6	1.1	0.28

a, b c = means within a row with different superscripts are different ($P< 0.05$)
SEM = Standard error of the mean
[1] control = atmospheric air
* SO_2 mean was higher than control mean in the cold environment ($P< 0.06$)

Figure 5.6 Nitric oxide production by alveolar macrophages (basal level of NO) that were not stimulated with LPS isolated from control steers and steers exposed to 1, 5 and 20 ppm of SO_2, thermo-neutral environment, n = 4, experiment 3

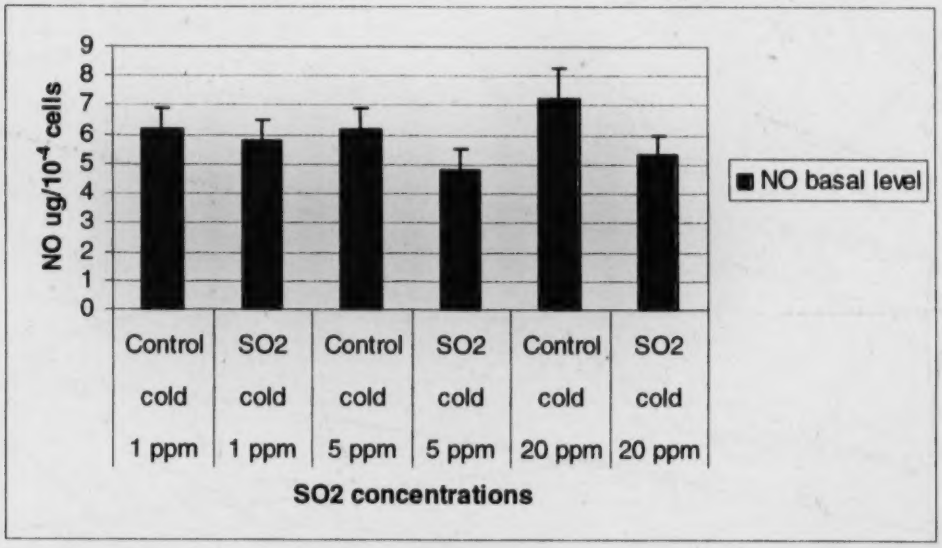
Bars comparing NO production within and between control steers and steers exposed to 1, 5, and 20 ppm of SO_2.



a b – Different letters denote significant differences between control group and groups exposed to SO_2, $P < 0.05$

Figure 5.7 Nitric oxide production by alveolar macrophages (basal level of NO) that were not stimulated with LPS isolated from control steers and steers exposed to 1, 5 and 20 ppm of SO_2, cold environment, n = 4, experiment 3

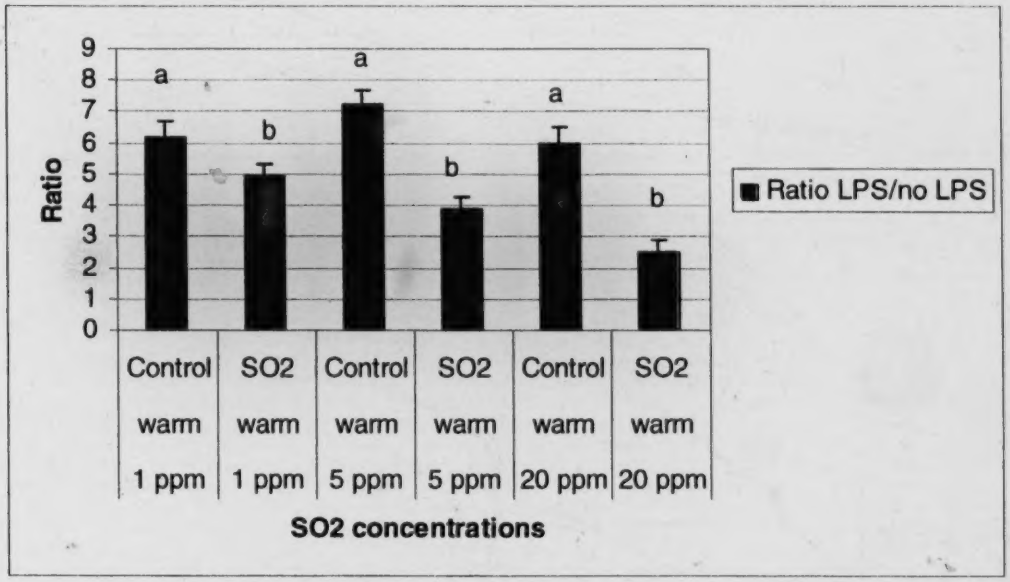
Bars comparing NO production within and between control steers and steers exposed to 1, 5, and 20 ppm of SO_2.



a b – Different letters denote significant differences between control group and groups exposed to SO_2, $P < 0.05$

Figure 5.8 Ratio of nitric oxide production by alveolar macrophages stimulated with LPS to non-stimulated (basal level of NO) macrophages isolated from BAL of control steers and steers exposed to 1, 5 and 20 ppm of SO_2, thermo-neutral environment, n = 4, experiment 3

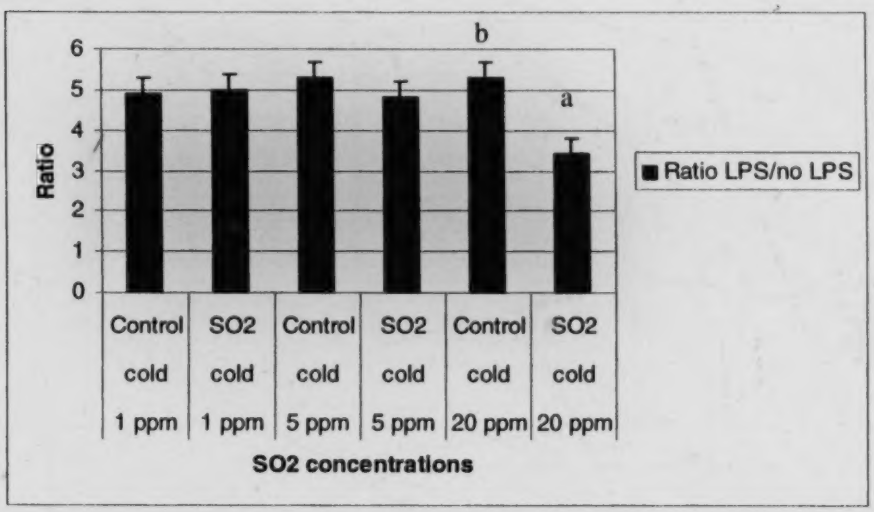
Bars comparing NO production within and between control steers and steers exposed to 1, 5, and 20 ppm of SO_2.



a b – Different letters denote significant differences between control group and groups exposed to SO_2, $P< 0.05$

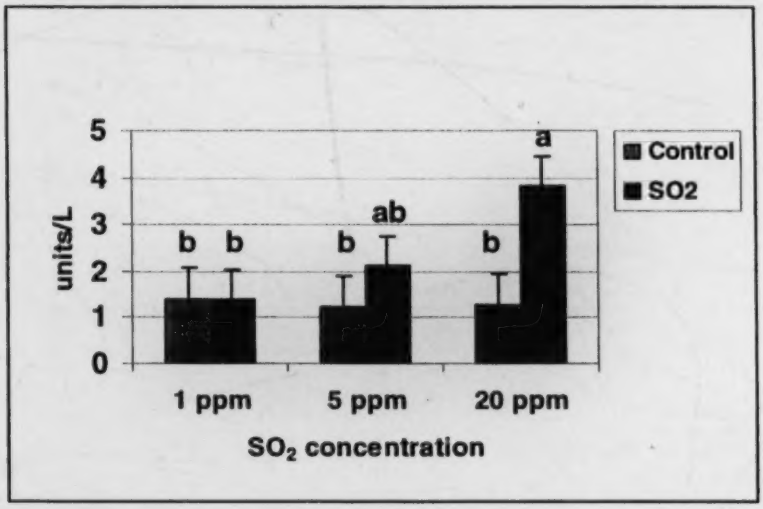
Figure 5.9 Ratio of nitric oxide production by alveolar macrophages stimulate with LPS to non-stimulated (basal level of NO) macrophages with LPS isolated from BAL of control steers and steers exposed to 1, 5 and 20 ppm of SO_2, cold environment, n = 4, experiment 3

Bars comparing NO production between control steers and steers exposed to 1, 5, and 20 ppm of SO_2.



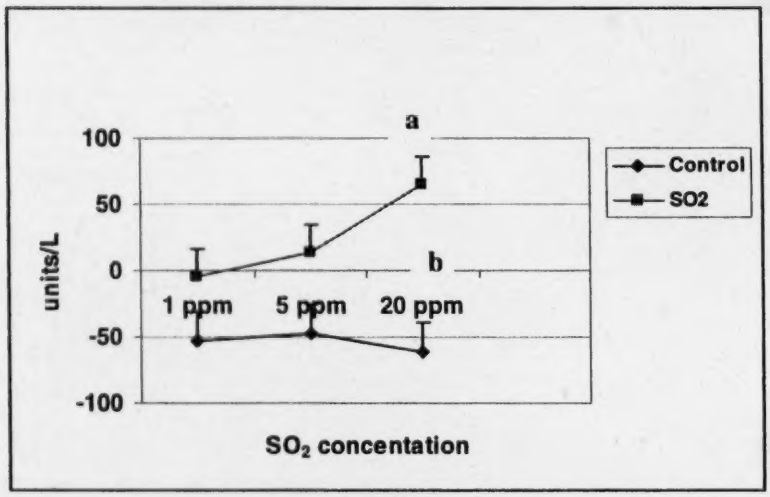
a b – Different letters denote significant differences between control group and groups exposed to SO_2, $P < 0.05$

Figure 5.10 Bar graph comparing activity of LDH in BAL of control steers and steers exposed to 1, 5, and 20 ppm of SO_2, n = 8, experiment 3



a b – Different letters denote significant differences between control group and groups exposed to SO_2, $P < 0.05$

Figure 5.11 The activity of ALP in BAL of control steers and steers exposed to 1, 5, and 20 ppm of SO_2. The activity of ALP in BAL samples is expressed as changes from the pre-exposure values, n = 8, experiment 3



a b – Different letters denote significant differences between control group and groups exposed to SO_2, $P < 0.05$

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Chapter 6

TOTAL CELL COUNT AND LEUKOCYTE PHENOTYPING IN PERIPHERAL BLOOD OF THE CATTLE EXPOSED TO DIFFERENT CONCENTRATIONS OF SULFUR DIOXIDE

INTRODUCTION

Sulfur dioxide (SO_2) is an important environmental pollutant (Alberta's Clean Air Act 1985). Epidemiological studies suggest that gas and oil well flaring have subtle health effects in cattle (Scott, 1998). The health and productivity of cow-calf herds with differing exposures to sour-natural-gas processing facilities were measured near Caroline, Alberta, in western Canada. The results demonstrated five examples of associations between increasing cumulative exposure to total sulfation and decreased productivity in the 18 models examined (Waldner *et al* 2001). However, the health effects of SO_2 in cattle based on controlled experiments are not known. This report is part of a large experimental study on the health effects of SO_2 in cattle. Sulfur dioxide gas dissolves in aqueous solutions into sulfite and bisulfite ions that are present in equilibrium (1:3) at neutral pH (Shapiro 1977). Limited information that is available on mechanism of SO_2 or sulfite/bisulfite toxicity in the body suggests that sulfur- and oxygen-centered free radicals formed in the process of sulfite/bisulfite oxidation are involved in oxidative damage of living cells and tissues (Meng and Zhang 1999). Reactive oxygen species (ROS) may affect cell metabolism and function at different levels including lipid peroxidation (Meng 2003) and DNA damage (Ueda and Shah 1992).

Components of the immune system are first to respond to the signals arising from stressed or damaged cells to facilitate protection of the organism. In previous chapters, it has been shown that SO_2 exposure damages pulmonary cells (Chapter 5) and decreases respiratory activity of neutrophils (Chapter 3), which are the first line of defense against infection. This study reports the effect of exposure to SO_2 on the total and differential white blood cell count, as well as on the different subtypes of leukocytes in cattle. Knowing the relative proportion of different subtypes of lymphocytes provides a more complete description of the immune system response than total number of WBC (Smith *et al* 1994).

Studies of immune cell subpopulations can be achieved by employing monoclonal antibodies (Mab) to surface antigens and determining of their phenotype (Dhabhar *et al* 1995). The surface antigens for lymphocyte subpopulations have been shown to be phenotypically and functionally homologous in different animal species and humans (Davis *et al* 1988). In cattle, WC (workshop cluster) is the nomenclature system for Mabs or antigens where homology with human CD (cluster of differentiation) antigens has not been established. Different subtypes of lymphocytes are involved in an integrated and regulated immune response to fight infection (Davis *et al* 1988). It is possible to detect Mabs that react with bovine surface immunoglobulins $(sIg)^+$ expressed on the acquired gated blood lymphocytes (Mukwedeya *et al* 1993) by using a flow cytometry that has been shown to be a rapid method for the identification of different leukocyte populations (lymphocytes, neutrophils, eosinophils, basophils, and monocytes) in bovine blood (Hageltorn and Saad 1986).

The hypotheses of the study were that inhalation of atmospheres with SO_2 could affect the peripheral blood cell count and the differentiation of blood leukocyte subpopulations in cattle. In addition, cold environmental temperatures such as those in Alberta winters may have interactive effects with SO_2.

The objectives of the study were to examine effects of atmospheric exposure to SO_2 on the differential integrity of immune cells in cattle.

MATERIALS AND METHODS

Four experiments were conducted in this study. For housing, feeding and management of the steers refer to Chapter 3.

Experiment 1 *(exposure to 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Two steers 18 months of age, with initial body weights of 635 kg and 615 kg, were used in this preliminary experiment. These animals were fed ration at a level calculated to provide approximately 1.6 times maintenance (NRC 1996). This experimental procedure was performed in a thermo-neutral environment (+17 to +20 °C).

Both steers were sequentially exposed to air with 5 ppm of SO_2 for a period of 7 days and with 20 ppm of SO_2 for 6 days. They had a 15-day recovery period after both, 5 and 20 ppm of SO_2 exposure.

Experiment 2 *(exposure to 0, 1, 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Twelve steers 6 to 9 months of age, with initial body weight averaging 391 kg were used in this experiment that was performed in a thermo-neutral environment (+17 to +20 °C). The animals were fed a ration at a level calculated to provide approximately 2.2 times their maintenance requirements (NRC 1996). A group of six steers was sequentially exposed to atmospheres with 1 ppm of SO_2 for 23 days, 5 ppm of SO_2 for 9 days, and 20 ppm of SO_2 for 8 days. A similar group of 6 control steers was exposed to ambient air ("0 ppm of SO_2") in hoods during the designated experimental exposure periods.

Experiment 3 *(exposure to 0, 1, 5, and 20 ppm of SO_2 in thermo-neutral and cold environments)*

Sixteen steers, 12 to 14 months of age, with initial body weight averaging 534 kg were used in this experiment. These animals were fed a ration at a level calculated to provide approximately 2.2 times their maintenance energy requirement (NRC 1996). A group of 8 steers was sequentially exposed to atmospheres with 1 ppm for 10 days, and then 5 and 20 ppm of SO_2 for 7 days at each level. This experiment was carried out simultaneously in two different thermal environments: thermo-neutral (+17 to +20 °C) and cold (-15 to -17°C) designated as "warm" and "cold" respectively. The steers and hoods were in a temperature-controlled chamber. These animals, unlike in the previous 2 experiments, were subjected to 2 different treatments. A similar group of 8 control steers was exposed to ambient air ("0 ppm of SO_2") during the designated exposure periods.

Duration of the SO_2 exposure

In all three experiments, the animals were exposed to SO_2 or clean air for 6 hours per day during five consecutive days. Within experiment 2 and 3, the steers were randomly allocated among experimental and control groups.

***Experiment 4** (effect of vitamin E and selenium supplementation)*

This is a follow-up experiment that was conducted with the steers previously exposed to 1, 5 and 20 ppm of SO_2 to examine the potential of nutritional treatments to reduce the impact of SO_2 (if any present) and enhance recovery in cattle. The experiment was performed after the end of each 20 ppm of SO_2 exposure period and lasted 14 days. A total of 14 steers from experiments 2 and 3 were used in experiment 4. Seven steers were given a single intramuscular (*m. glutaeus medius*) injection of an emulsion of selenium-α-tocopherol (E-Sel injection, DIN 01940279, Citadel Animal Health, Edmonton, AB). The dose was calculated as 45 µl per kg of body weight providing supplemental vitamin E as dl-alpha-tocopheryl acetate (3.2 IU/kg) and selenium as sodium selenite (0.05 mg/kg). Another group of 7 control steers received no vitamin E and selenium supplementation.

Initial values of the tested parameters

A pre-exposure period of 14 days in duration was conducted at the beginning of each experiment, in order to obtain the initial values for the tested parameters, as well as to evaluate the initial health status of the steers. During this period [study days (-14) to (0)], in which a thermo-neutral and cold environment (where applicable) were maintained in the temperature controlled chambers the steers were placed in hoods for 6 h per day and ventilated with fresh air.

Sample collection

Blood samples

Blood samples for hematology and lymphocytes studies were collected by venipuncture (external jugular vein) into Beckman vacutainer test tubes filled with EDTA in the morning. Hematological tests were performed once each week and lymphocyte phenotyping assays once every two weeks. To avoid the possibility diurnal variations,

blood was always collected and the procedures were always performed at the same time of the day – 08:00.

Hematological parameters

Red blood cells (RBC) and white blood cell (WBC) numbers were determined with an electronic cell counter (Coulter Model S-Plus IV). Blood smears were stained with Wright - Giemsa using an automated stainer (Hema-Tek, Miles Laboratories). Blood smears were examined by light microscopy, where 100 cells or 1 % of WBC was counted to derive the relative WBC differential counts. Serum biochemical parameters including total iron and unsaturated iron binding capacity were determined on an automated multiple-channel analyzer (Hitachi 704 Chemistry Analyzer, Boehringer Mannheim) using recommended reagents and analytical methods. Calibration standards were included in each assay.

Mononuclear cell phenotyping

Identification of lymphocyte subpopulations in whole blood were performed by using fluorescent conjugated Mab (Table 6.1) purchased from Veterinary Medical Research & Development (VMRD) Inc. and a Becton-Dickinson FACScan flow cytometer.

Table 6.1 The monoclonal antibodies (Mab) as markers for identification of ruminant peripheral blood leukocyte subpopulations that were used in immuno-assays

Mabs	Dilution	Subpopulations	Cell line	Isotype	Fluorochrome
CD2	1:100	Mature T lymphocytes	B26A4	IgM	PE
CD3	1:100	T lymphocytes	MM1A	IgG1	FITC
CD4	1:100	"helper" T lymphocytes (Th)	GC50A1	IgM	PE
CD8	1:100	Cytotoxic T lymphocytes (Tc)	BAQ111A	IgM	PE
CD25	1:60	IL-2	CACT116A	IgG1	FITC
TcR1	1:200	αβ-T lymphocyte receptors	86D	IgG1	FITC
WC1-N1	1:100	γδ-T lymphocyte receptors	B7A1	IgM	PE
B-B2	1:100	B cells	BAQ44A	IgM	PE
CD14	1:100	Monocytes	CAM36A	IgG1	FITC

This test system evaluated the changes and the distribution of leukocyte subpopulations in whole blood by using single fluorescence analyses to determine whether a given Mab recognizes a molecule expressed on one or more lineages of leukocytes. The method comprised of adding specific monoclonal antibodies (mouse anti-bovine) to blood cells and affinity-isolated antibodies specific to mouse immunoglobulins (Igs) (goat anti-mouse) reacted and conjugated with fluorochromes FITC -- fluorescein isothiocyanate (Cedarlane Laboratories Limited, catalog M32101) and PE -- phycoerythrin (Cedarlane Laboratories Limited, catalog M31604). The samples than were analyzed by FACScan flow cytometry (Becton-Dickinson, Sunnyvale, CA) according to the relative fluorescence intensity using CellQuest software. Resulting percentages of cells were corrected for background fluorescence (0-5%) determined by incubating the cells with appropriate isotype controls (IgG1, IgM). The immuno-phenotyping of lymphocyte subsets may be influenced by a variety of technical factors, which include the duration and temperature of sample storage and the method used for staining samples (Ekong et al 1993). Therefore, monoclonal antibodies were optimized prior to use and diluted for the assay accordingly (Table 6.1).

The procedure for preparing bovine leucocytes for the leukocyte immunophenotyping assay was previously described at Washington State University (Dr. W. Davis, personal communications). Gamma-globuline (GG)-free horse serum for wash buffer was purchased from Gibco (Burlington, ON). Microplates (96-v-bottom well) and Falcon immunoassay tubes were purchased from Fisher Scientific (Edmonton, AB, Canada), and chemicals for buffers and solvents were purchased from Sigma chemicals (St. Louis, MO).

Erythrocytes were lysed by the mixing 8 ml of whole blood with 2 ml acid citrate dextrose (ACD) anti-coagulant and added to 40 ml of warm (37°C) tris-ammonium chloride (pH 7.2) lysis buffer and shaking in a water bath for 7 min. The white cells were pelleted by centrifugation at 1000 rpm for 7 min and washing 2 times with wash buffer [phosphate buffered saline (PBS) containing 10% v/v 1x ACD, 2% v/v horse serum, and 1% v/v of NaN_3 (2% in PBS)]. The cells were separated from the washing fluid by centrifugation for 3 min at 1000 rpm. After washing, leucocytes were resuspended in 1.5 ml of wash buffer and stored at 4°C for 24 hours. Fifty µl suspension of these cells (5 x 10^5 cells) were used in the immunofluorescence assay. ACD anticoagulant was proposed to enhance leukocyte subpopulation separation compared to ethylenediamine tetra-acetic acid (EDTA), heparin and sodium citrate (Abella et al 1994). Dr. Abella and coworkers also suggested that for isolation of WBC, the whole blood lysis method was faster and gave the same results as Ficoll gradient separation 1.077 and 1.083. In addition, they found that blood samples collected in ACD could be kept at 22°C or at 4°C for up to 32 hours before phenotyping. In this study, WBC were isolated immediately after the blood collection and then isolated cells were kept the suspended in ACD buffer at 4°C for 24 hours before immunophenotyping. After conjugation with FITC or PE, WBC were fixed with 2% v/v paraformaldehyde in PBS, and were stored at 4°C for subsequent analysis by flow cytometry.

Mononuclear cell phenotyping

Fifty µl of cells were added to each well of a non-sterile 96-well v-bottom microtiter plate as per the procedure described in Chapter 5. The cells were incubated

with appropriately diluted monoclonal antibodies for 30 minutes in the dark, on ice. Plates were washed 3 times with phosphate buffered saline containing fetal calf serum (40 g/L; wash buffer). After each wash the plates were centrifuge at 1000 rpm for 1 min. After addition to the wells of appropriately diluted fluorochromes FITC or PE the plates again were incubated for 30 minutes in a dark, on ice. The plates were washed 2 times with wash buffer, with following centrifugation at 1000 rpm for 1 min after each wash. Conjugated with appropriate monoclonal antibody and FITC or PE cells were fixed in phosphate buffered saline containing 2% paraformaldehyde, covered with foil and stored at 4°C for subsequent analysis. The well contents were transferred to the test tubes and analyzed on a Facscan (Bacton-Dickinson, Sunnyvale, CA) according to the relative fluorescence intensity using Cell Quest software. Resulting percentages were corrected for background fluorescence (0-5%) determined by incubating the cells with appropriate isotype controls (IgG1 or IgM).

Statistical analyses

Two steers in sequence were exposed to air with SO_2 in experiment 1, therefore, they both were designated as "treated" animals. The results across the exposure periods [pre-exposure (0ppm), 5 ppm, after 5 ppm, 20 ppm and after 20 ppm] are presented as arithmetic means of 2 steers. The steers in experiments 2 and 3 were grouped in blocks involving similar treatments, maintenance, and control conditions since only a few animals could be exposed to SO_2 simultaneously. The research study was performed using a factorial experimental design (treated versus control animals). For the purpose of statistical analysis, we considered animals exposed to 0 and 1 ppm levels, 0 and 5 ppm levels, 0 and 20 ppm levels of SO_2 as separate groups, and carry over effect of the treatments could not be determined. Data were analyzed using Proc GLM with fixed effect of treatments [SO_2 and control (no SO_2)], environment (warm and cold) where applicable, and two blocks. In experiments 2 and 3, the pre-exposure values for all dependent variables were included as a covariate. In experiment 4 the data were compared with the values that were obtained at exposure steers to 20 ppm level of SO_2.

Least Square Means (Lsmeans) were estimated using the Pdiff option (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA).

RESULTS

Experiment 1

Hematological parameters

The blood cell numbers (#/mm^3) were within the expected normal range for cattle and were not different between the SO_2 treatments. The data with a reference to normal values are listed in the Appendix (Table AP2). However, the neutrophil numbers appeared to have a trend to decrease from the pre-exposure period to the period of exposure steers to 20 ppm of SO_2 by 35%, and remained low for up to 14 days after the exposure steers to 20 ppm of SO_2.

Experiment 2

Hematological parameters

There were no significant effects of SO_2 exposures on the complete blood count parameters (Appendix, Table AP3). The blood neutrophil numbers were not significantly influenced ($P > 0.05$) by SO_2 (Figure 6.1), although there was a non-significant trend to decrease in neutrophil numbers after exposure of animals to 20 ppm level when compared to control animals. The neutrophil numbers were slightly, but not significantly, higher after exposure to the 1 and 5 ppm levels.

Mononuclear cell phenotyping

The data on leukocyte subtypes determined by monoclonal antibodies and flow cytometry are provided in Table 6.2. Significant changes ($P < 0.05$) were observed for certain subpopulations of cells. In particular there was a significant ($P < 0.05$; $P < 0.01$) decrease of 16, 13 and 16% in the proportion of $CD4^+$ (T-helper lymphocytes) and a significant ($P < 0.05$) increase of 27, 50 and 58% in the proportion of $CD8^+$ (cytotoxic T

lymphocytes) at 1, 5 and 20 ppm of SO_2 exposure levels, respectively. A significant ($P< 0.01$) increase of 16, 28 and 55% was observed in the proportion of $CD25^+$ cells (marker for interleukin-2 receptor) and increase of 29, 28 and 55% in the proportion of $TcR1^+$ (T-cell receptor) ($P< 0.05$) at 1, 5 and 20 ppm of SO_2 exposure levels compared to controls. Consistent significant ($P< 0.01$) increases of 23, 83, 43% were observed in the proportion of $CD14^+$ (monocytes/ macrophages) at all 3 levels of SO_2 exposure.

The ratio of CD4 to CD8 T lymphocytes was significantly (< 0.05) decreased by 40 – 45% at all levels of SO_2 exposure in steers compared to their corresponding control animals (Table 6.3).

Experiment 3

Hematological parameters

There were no effects of SO_2 exposure on hematology parameters (Appendix, Table AP4). Assessments of WBC numbers and differential WBC count did not show any changes from the pre-exposure values of these parameters in exposed and in control steers, or between control and SO_2 treated animals. Also within the expected normal range for cattle there was a significant ($p < 0.05$) increase in neutrophil numbers in response to 1 ppm of SO_2 in the warm but not cold environments (Figure 6.2). There was a tendency for neutrophil number to decrease after exposure of steers to 20 ppm of SO_2 in both warm and cold environments, although this was not significant due to large variation between animals (Figure 6.2).

Mononuclear cell phenotyping

Table 6.4 shows that the proportion of $CD4^+$ cells was significantly ($P< 0.01$) decreased by 21, 50 and 34% in the warm environment and by 14, 44 and 21% in the cold environment after exposure of steers to 1, 5 and 20 ppm of SO_2. Similar to the results of experiment 2, the proportion of $CD8^+$ cells was increased by 40, 35 and 41% in the warm environment and by 57, 17 and 43% in the cold environment after exposure steers to 1, 5 and 20 ppm of SO_2, respectively. $CD25^+$ cells significantly ($p<0.05$) increased by 22, 17 and 56% in the warm environment and by 12, 16 and 43% in cold environment at all

three levels of SO_2, respectively. The proportion of $TcR1^+$ cells was significantly ($P< 0.01$) increased on average by 25, 24 and 30% in warm and cold environments after exposure of steers to 1, 5 and 20 ppm of SO_2, respectively. The expression of $CD14^+$ marker in response to SO_2 was consistent with the result of experiment 2 and the proportion of $CD14^+$ cells was significantly ($p<0.05$) increased on average by 25, 40 and 60% in both warm and cold environments. There were no significant changes shown for the other blood cell subtypes (CD2, WC1 and B cells) due to SO_2

Ratio of CD4 to CD8 T lymphocytes was significantly (< 0.05) decreased by 40 – 45% at all levels of SO_2 exposure in warm and cold environments in steers compared to their corresponding control animals (Table 6.5).

Experiment 4

Mononuclear cell phenotyping

Patterns of leukocyte subtypes (Figure 6.3) were not affected ($P> 0.05$) by vitamin/selenium treatment following the end of exposure to a concentration of 20 ppm SO_2.

DISCUSSION

The blood neutrophils are phagocytic cells that are involved in innate immune defense processes (Minatel and Carfagnini 2000). The change in neutrophil numbers was not consistent across all experiments. In experiment 1, neutrophil numbers tended to gradually decrease as a result of exposure to increasing levels of SO_2. Although similar trends for a decrease in neutrophil numbers appeared as a response to exposure to 20 ppm of SO_2 in experiments 2 and 3, the results were not significant due to the large variability among animals within treatments. An inflammatory response to SO_2 was expected in the tissues of the pulmonary system and leukocyte trafficking into pulmonary tissue also was expected to occur as a component of the host defense response (Wagner and Roth 2000). Consequently, our observations support the assumption that some neutrophils migrated from the blood to the sites, e.g. the lungs where inflammatory or other cytotoxic processes may have been occurring. The increased ratio of degenerative to healthy

pulmonary cells and increased percentage of pulmonary neutrophils detected in the BAL fluids from steers exposed to SO_2 (Chapter 5), support the suggestion that SO_2 has cytotoxic effects on the cells of the pulmonary system. It was also found that the proportion of $CD14^+$ cells (marker for monocytes/macrophages) in peripheral blood of steers exposed to 1, 5, and 20 ppm levels of SO_2 in both warm and cold environments was significantly higher compared to control animals. It is possible that this increased percentage of blood monocytes may have been induced by mediators arising from the damaged pulmonary cells (Nathan 1987).

The data on leukocyte subtypes determined by use of monoclonal antibodies and flow cytometry showed significant changes in certain subpopulations of blood cells. In particular, there was evidence for a decrease in $CD4^+$ (T-helper lymphocytes) ($P< 0.05$; $P< 0.01$) and an increase in $CD8^+$ (cytotoxicauppressor T lymphocytes), possibly in response to cytotoxic effects of SO_2. The decrease in ratio of circulating lymphocytes in the helper (CD4) to suppressor/cytotoxic (CD8), a measure of cell mediated immunity, indicate that regulatory signals that favor development of T lymphocytes into cytotoxic $CD8^+$ cells are enhanced. It is probable that this occurs in response to signals arising from exposure of pulmonary tissue (Mcinnes *et al* 1999) to SO_2. Also it has been shown that activated $CD8^+$ T lymphocytes may play an important role in the regulation and expression of the local immune response to pathogens (Park *et al* 1992). In the present study, the steers appeared to be free from infection that could subsequently result in pneumonia. The increased proportion of $CD25^+$ cells ($P< 0.01$) from 20% (at 1 and 5 ppm of SO_2) up to 50% (at 20 ppm of SO_2) in both environments relative to controls is indicative of an increased expression of a receptor for interleukin-2 (IL-2) cytokine on T lymphocytes. Since IL-2 is produced during the early stages of the immune response it facilitates proliferation of most lymphocyte subtypes (Swain 1991). In general, the IL-2 cytokine normally signals the immune system to increase the production of $CD4^+$ lymphocytes (Ayoub and Yang 1995), therefore, an increase in IL-2 receptor expression may prepare the system to eventually generate more $CD4^+$ cells as a result of their depletion found in our study. However, we did not measure the concentration of IL-2 cytokine. The decreased percentage of $TcR1^+$ (T-cell receptor) ($P< 0.05$) is consistent with the decrease actually observed in the $CD4^+$ lymphocyte population expressing

alpha/beta TcR1 surface antigen receptors (Clevers *et al* 1990). The relatively constant expression of CW-1$^+$ (gamma/delta) T cells, indicates that immune cell changes which mainly occur in the gastrointestinal tract (Ayoub and Yang 1995, Clevers *et al* 1990, Crocker G *et al* 1993), were not affected by SO_2 treatments of the steers. The changes that were observed in subtype patterns among the immune cells may have been a result of the pulmonary cell damage induced by SO_2 in lung tissues (Chapter 5). This is supported by the consistent increases, observed for CD14$^+$ cells ($P< 0.01$) at all three levels of SO_2 exposure and in both cold and warm environments. The increased proportion of CD14 surface antigen receptor may be an indication that the signals arising from the affected lung tissue are inducing monocyte/ macrophage mobilization to deal with the pulmonary damage (Blusse van Oud *et al* 1981).

Patterns of changes in leukocyte subtypes in blood of steers were not affected by vitamin/selenium treatment following the end of exposure to 20 ppm of SO_2 even though the treatments increased plasma vitamin E concentration (Chapter 4). The persistent increase in expression of the (monocyte/macrophage) CD14$^+$ marker indicates that exposure to 20 ppm of SO_2 caused the immune system to stimulate proliferation of phagocytic cells in the peripheral blood of the steers, which continued to respond to *in vivo* signals even 14 days after the end of SO_2 exposure.

Figure 6.1 Neutrophil numbers in peripheral blood of control steers and steers exposed to the 0, 5, and 20 ppm levels of SO_2. n= 6, experiment 2

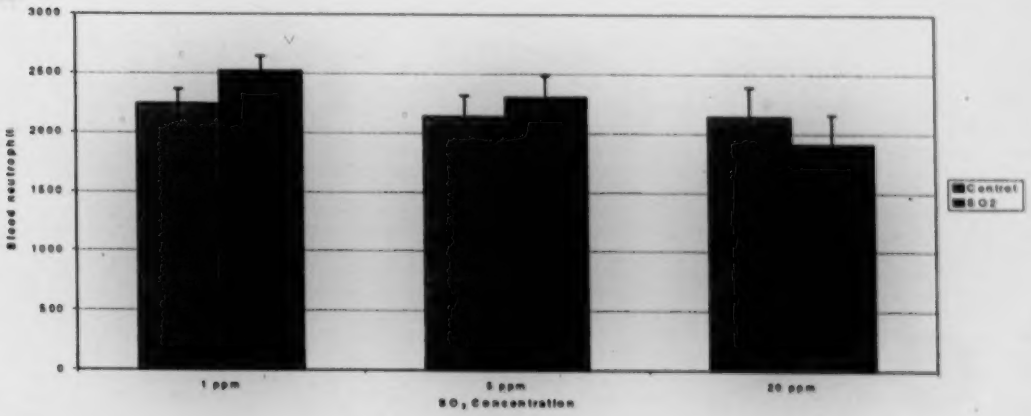


Table 6.2 Lymphocyte subtypes (% gated) in blood from control steers and from steers exposed to various concentrations of SO_2. n = 6, experiment 2

Sub-type	SO_2 level	Control[1]	SO_2	SEM
CD2				
	1	56	58	1.94
	5	60	59	2.74
	20	66	56	2.74
CD4				
	1	38^a	32^b	1.07
	5	39^a	34^b	1.51
	20	38^a	32^b	1.51
CD8				
	1	11^b	14^a	0.44
	5	12^b	18^a	0.63
	20	12^b	19^a	0.63
CD25				
	1	25^b	29^a	0.91
	5	29^b	37^a	1.28
	20	29^b	45^a	1.28
TcR1				
	1	17^b	24^a	0.93
	5	15^b	24^a	1.27
	20	14^b	23^a	1.27
WC-1				
	1	24	26	1.16
	5	27	25	1.62
	20	25	27	1.62
B cells				
	1	42	44	1.23
	5	42	43	1.73
	20	43	44	1.73
CD14				
	1	22^b	27^a	0.77
	5	23^b	42^a	1.09
	20	23^b	33^a	1.09

a b =- means within the row with the same letter are not significantly different ($P< 0.05$)
[1] control = atmospheric air
SEM = standard error of the mean

Table 6.3 The ratio of helper (CD4) to cytotoxic/suppressor (CD8) T lymphocytes in peripheral blood of control steers and steers exposed to 1, 5 and 20 ppm of SO_2, n = 6, experiment 2

SO_2 level (ppm)	CD4/CD8 ratio		SEM
	[1]Control	SO_2 treated	
1	3.6[a]	2.3[b]	0.17
5	3.5[a]	2.1[b]	0.25
20	3.2[a]	1.8[b]	0.25

a b - means within the row with the same letter are not significantly different ($P< 0.05$)
[1] control = atmospheric air
SEM = standard error of the mean

Table 6.4 Lymphocyte subtypes (% gated) in blood from control steers and from steers exposed to various concentrations of SO_2 in warm and cold environments, n = 4, experiment 3

SubType	SO2 Level (ppm	Warm		Cold		SEM
		Control	SO2	Control	SO2	
CD2	1	65	63	65	64	1.71
	5	70	66	60	58	1.59
	20	50	50	50	52	1.59
CD4	1	29^a	23^b	28^a	24^b	1.30
	5	30^a	15^b	27^a	15^b	1.51
	20	29^a	19^b	28^a	22^b	1.51
CD8	1	20^b	28^a	21^b	33^a	1.28
	5	23^b	31^a	23^b	27^a	1.46
	20	22^b	31^a	21^b	32^a	1.46
CD25	1	32^b	39^a	33^b	37^a	1.48
	5	35^b	41^a	37^b	43^a	1.70
	20	25^b	39^a	28^b	40^a	1.70
TcR1	1	22^b	28^a	22^b	27^a	0.92
	5	23^b	29^a	23^b	28^a	1.07
	20	22^b	29^a	22^b	28^a	1.07
WC-1	1	25	24	23	23	0.95
	5	25	24	26	24	1.06
	20	25	25	24	23	1.06
B cells	1	54	55	56	55	1.32
	5	55	55	55	54	1.52
	20	53	53	54	53	1.52
CD14	1	15^b	18^a	16^b	20^a	0.77
	5	16^b	22^a	14^b	22^a	1.09
	20	14^b	24^a	15^b	23^a	1.09

a b - means within the row with the same letter are not significantly different ($P< 0.05$)
[1] control = atmospheric air
SEM = standard error of the mean

Table 6.5 The ratio of helper (CD4) to cytotoxic/suppressor (CD8) T lymphocytes in peripheral blood of control steers and steers exposed to 1, 5 and 20 ppm of SO_2 in warm and cold environments, n = 4, experiment 3

SO2 Level (ppm)	CD4/CD8 ratio				SEM
	Warm		Cold		
	Control	SO_2	Control	SO_2	
1	1.5^a	0.8^b	1.4^a	0.7^b	0.13
5	1.3^a	0.5^b	1.2^a	0.5^b	0.88
20	1.4^a	0.6^b	1.3^a	0.7^b	0.88

a b - means within the row with the same letter are not significantly different ($P< 0.05$)
[1] control = atmospheric air
SEM = standard error of the mean

Figure 6.2 Neutrophil numbers in peripheral blood of control steers and steers exposed to 0, 1, 5, and 20 ppm levels of SO_2 in warm and cold environments, n = 4, experiment 3.

a b - means within the row with the same letter are not significantly different ($P< 0.05$)

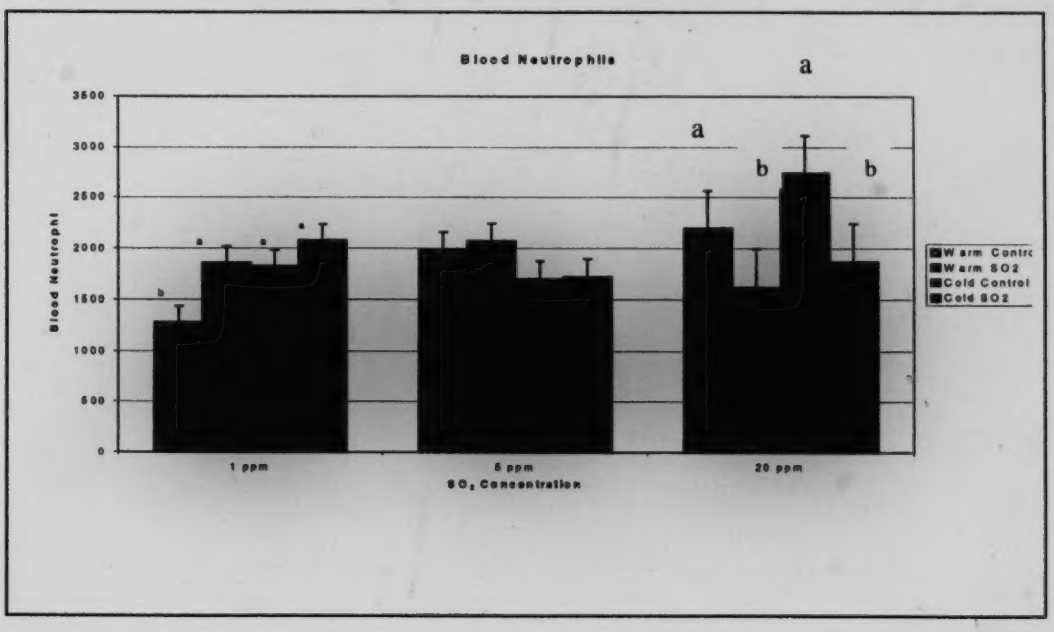
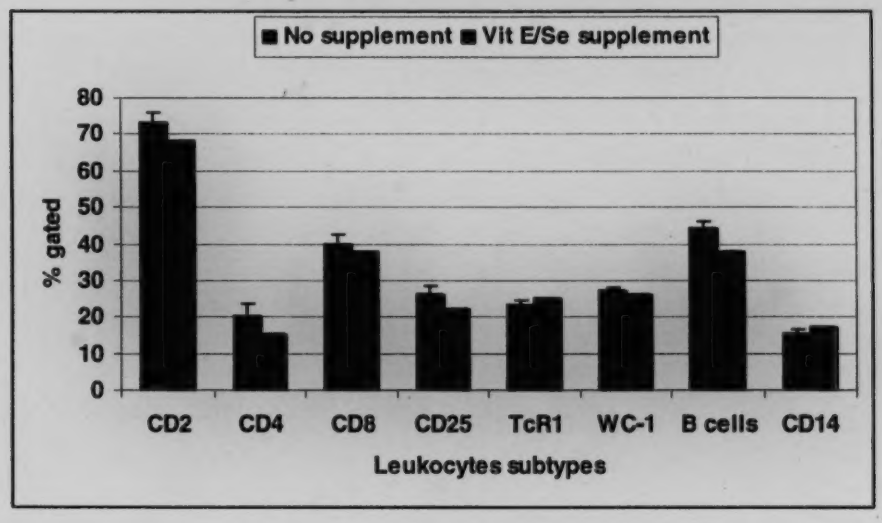


Figure 6.3 Leukocyte subtypes (% gated) in blood from control steers (no supplement) and steers treated with vitamin E and selenium during a two-week period following the end of exposure to 20 ppm of SO_2.The values obtained after the supplementation were compared to the values obtained after exposure steers to 20 ppm of SO_2 level, n = 7, experiment 4



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Chapter 7

EFFECT OF SULFUR DIOXIDE ON ENERGY EXPENDITURE, HEART RATE, RESPIRATORY FREQUENCY, RECTAL TEMPERATURE AND SERUM CORTISOL IN CATTLE

INTRODUCTION

Sulfur dioxide (SO_2) is a major air pollutant worldwide and has particular relevance to Alberta. As a result of ancient sedimentation, compression and fermentation of organic matter in the soil, fossil fuel deposits were created throughout the province. As of 1994 a total of about 167,000 oil and gas wells had been drilled (Scott 1998). The raw gas processing capacity in the province in 1994 was over 1 billion m^3 of gas per day, including licensed processing capabilities of up to 32091 tonnes (t) of sulfur per day (Scott 1998). Over 65,000 gas and oil wells remained active as of 1996 (Scott 1998). The total industrial emissions of SO_2 in the province of Alberta, Canada in 1993 were 600,000 t per year (CASA 1997).

Humans and animals are exposed daily to a wide variety of environmental xenobiotics or air pollutants that are inhaled, ingested or absorbed by the skin or by mucous membranes. Some xenobiotics are innocuous, but many can provoke physiologic responses. Five major substances contribute to about 98% of air pollution, including sulfur oxides (about 14%) (Katzung 1997). Sources of SO_2 in Alberta are human activities including combustion of sulfur-containing fossil fuels, coal-fired electrical generation, emissions from sour gas flares, sulfur recovery plants, and recovery of oil from tar sands. There is concern that air pollutants (sour gas) may have adverse effects on livestock (AEC 1992). Also an excessive level of sulfur-containing compounds in domestic ruminant rations, (e.g. the SO_2 treated silage) has been found to be associated with polioencephalomalacia, a brain disease associated with a deficiency of thiamine (Olkowski 1997).

SO_2 is rapidly hydrated forming sulfite (SO_3^{2-}), which, in reaction with superoxide, forms a very reactive bisulfite radical ($HSO_3^{\bullet -}$) and hydrogen peroxide (H_2O_2). $SO_3^{\bullet -}$ participates in several radical chain processes such as lipid peroxidation (Hippeli and Elstner 1995). A study on Swiss-albino male rats showed that SO_2 inhalation enhances

lipid peroxidation and influences antioxidant enzymes of erythrocytes (Gumuslu-Saadet et al. 1998). A combination of SO_2 with soot or asbestos (asbestos is a very inert material) is very toxic and may contribute to certain respiratory disorders (Hippeli and Elstner 1995).

Stress is a stimulus that results in the adaptation of an animal to the environment (Christopherson and Thompson 1983) and may influence the metabolism of the animal. An animal can be subjected to various kinds of stressors produced by thermal imbalances, husbandry practices and management, transportation, environmental pollution, ionizing radiation, and chemical intoxication, which may increase an animal's susceptibility to infectious diseases (Breazile 1988; Blecha and Charley 1990). In evaluating stress in farm animals, endocrine responses such as adrenal cortisol secretion are quite variable, and may be increased in response to acute stress (Cooper *et al* 1995, Graham *et al* 1981).

Temperatures outside the animals' thermoneutral zone and nutritional imbalances may also add stress to an animal that is in distress from intoxication. The dose-response interactions between toxicological effects of SO_2 and thermal stress are not known in cattle. A reasonable assumption is that the stressful components are additive. However, potentiation and antagonism could also occur. It is known that the minute ventilation of the animal is directly proportional to the rate of metabolism (Reece 1997). At low temperatures metabolism is increased, respiratory frequency is decreased, tidal volume is increased and, as a result, the minute lung ventilation may be increased (Reece 1997). A study on rodents shows that at low temperature these respiratory and tidal volume changes increased the uptake of toxic materials by the animal (Watkinson *et al* 1997).

Environmental temperatures may affect body metabolism resulting in alteration of some physiological parameters including energy requirements (Graham and Christopherson 1981). Body weight gains, rectal temperatures, respiratory frequencies and water intakes were significantly lower in young calves housed at colder temperatures (-4°C) compared to animals housed at 10°C. Calves housed at -4°C had also higher maintenance energy requirements (0.133 Mcal metabolizable energy/kg^{75}) than calves housed at 10°C (0.101 Mcal metabolizable energy/kg^{75}) (Scibilia *et al* 1987). In older cattle, the energy expenditure has been shown to increase by 20% for yearling steers when exposed to -11°C (Miaron *et al* 1995). In two-year-old steers, exposure to -20°C

increased energy expenditure by approximately 8-10% (Hidari *et al* 1991). While the effects of cold stress are known, the effect of chemical stress, such as SO_2 pollution, on energy metabolism in humans and animals is still largely uncharacterized. Also, since no data exist on dose-responses of cattle to SO_2, there was a need to study the effect of controlled exposure of cattle to varying concentrations of SO_2.

The objective was to test the hypothesis that SO_2 acts as a respiratory irritant in cattle and that cold stress and SO_2 exposure will have interactive effects to influence metabolic and physiological processes including circulating cortisol and energy expenditure. Another objective was to identify concentrations and cumulative doses of atmospheric SO_2 that are toxic to cattle.

MATERIALS AND METHODS

Four experiments were conducted in this study. For housing, feeding and management of the steers refer to Chapter 3.

Experiment 1 *(exposure to 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Two steers 18 months of age, with initial body weights of 635 kg and 615 kg, were used in this experiment. These animals were fed ration at a level calculated to provide approximately 1.6 times maintenance (NRC 1996). This experimental procedure was performed in a thermo-neutral environment (+17 to +20 °C). Both steers were sequentially exposed to air with 5 ppm of SO_2 for a period of 7 days and with 20 ppm of SO_2 for 6 days. They had a 15-day recovery period after both, 5 and 20 ppm of SO_2 exposures.

Experiment 2 *(exposure to 0, 1, 5 and 20 ppm of SO_2 in thermo-neutral environment)*

Twelve steers 6 to 9 months of age, with initial body weight averaging 391 kg were used in this experiment that was performed in a thermo-neutral environment (+17 to +20 °C). The animals were fed a ration at a level calculated to provide approximately 2.2

times their maintenance requirements (NRC 1996). A group of six steers was sequentially exposed to atmospheres with 1 ppm of SO_2 for 23 days, 5 ppm of SO_2 for 9 days, and 20 ppm of SO_2 for 8 days. A similar group of 6 control steers was exposed to ambient air ("0 ppm of SO_2") in hoods during the designated experimental exposure periods.

Experiment 3 *(exposure to 0, 1, 5, and 20 ppm of SO_2 in thermo-neutral and cold environments)*

Sixteen steers, 12 to 14 months of age, with initial body weight averaging 534 kg were used in this experiment. These animals were fed a ration at a level calculated to provide approximately 2.2 times their maintenance energy requirement (NRC 1996). A group of 8 steers was sequentially exposed to atmospheres with 1 ppm for 10 days, and then 5 and 20 ppm of SO_2 for 7 days at each level. This experiment was carried out simultaneously in two different thermal environments: thermo-neutral (+17 to +20 °C) and cold (-15 to -17°C) designated as "warm" and "cold" respectively. The steers and hoods were in a temperature controlled chamber. These animals, unlike in previous 2 experiments, were subjected to 2 different treatments. A similar group of 8 control steers was exposed to ambient air ("0 ppm of SO_2") during the designated exposure periods.

Duration of the SO_2 exposure

In all 3 experiments, the animals were exposed to atmospheres with SO_2 or ambient air for 6 hours/ day for 5 consecutive days each week. Within experiments 2 and 3, the steers were randomly allocated to experimental and control groups.

Initial Values

A pre-exposure period of 14 days in duration was conducted at the beginning of each experiment, in order to obtain the initial values for the tested parameters, as well as to evaluate the initial health status of the steers. During this period [study days (-14) to (0)], in which a thermo-neutral and cold environment (where applicable) were maintained

in the temperature controlled chambers, the steers were placed in hoods for 6 h per day and the hoods were ventilated with fresh air.

Sample Collection

Blood sampling

Blood samples for complete blood count (CBC) were collected into vacutainer tubes with EDTA, and, for blood serum biochemistry, into serum separator vacutainer tubes. The samples were obtained once each week at 08:00 hours by venipuncture. In experiments 1 and 2 the samples were analyzed at the Veterinary Pathology Laboratory (VPL, Edmonton, Alberta, Canada) and in experiment 3 by the Toxicology Business Unit at the Alberta Research Council (ARC, Vegreville, Alberta, Canada).

Red blood cell numbers (RBC), hematocrit (Hct), hemoglobin (Hb) levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), white blood cell numbers (WBC), platelet numbers (Plt) and mean platelet volume (MPV) were done by automated procedures (Coulter Model S-plus IV, Coulter Electronics, Miami, Florida, USA). Blood smears were stained with a modified Wright-Giemsa stain (Ames Hema – Tek Slide Stainer, Bayer Health Care, Toronto, Ontario). The differential counts for leukocyte numbers were determined by morphologically evaluating and classifying 2% percent of the WBC. The absolute numbers of neutrophils (Neu), band neutrophils (Bands), lymphocytes (Lympho), monocytes (Mono), basophils (Baso), and eosinophils (Eosin) were derived by multiplication of the differential count percentage by the WBC. The Neu and Lympho were used to calculate the neutrophil/lymphocyte (N/L) ratio (see Appendix B).

Serum chemical parameters (See list of the parameters in the Appendix C) were determined on an automated multiple-channel analyzer Hitachi 704 (Hitachi, Roche Diagnostics, Laval, Quebec).

For cortisol RIA in experiment 1, blood samples were collected from the steers through a jugular catheter every hour during a 5-hour period. A jugular catheter was placed in the external jugular vein 2 hours before the first blood sample was collected. The first sample was collected before the SO_2 exposure, and the rest of the blood samples

were collected at hourly intervals during the SO_2 exposure. In experiment 3, blood samples for cortisol were collected at only 3 times starting 2 hours after catheter insertion and 1 hour after SO_2 exposure began.

Clinical observations

Twice a week skin, hair, eyes, and eyelid, mouth and nose mucous membranes were evaluated for any lesions. Also at these times the chest of each animal was auscultated for abnormal respiratory sounds. Occurrence of pathological sounds in lungs and lesions on skin and mucous membranes were recorded only as "present" or "absent."

Body weight (BW)

The steers were weighed 2 times a week at 08:00 hours using an electronic scale.

Heart rate (HR)

Heart rate was recorded twice a week with a physiological recorder (Beckman®, R511A, USA) connected to 3 electrodes located on the back and two sides of the animal body. The recordings were 5 min long performed 3 times during a 6-hour exposure run: at the beginning, at 3 hours and at the end of the SO_2 exposure run.

Respiratory frequency (RF)

RF was recorded twice a week by observing respiration and recorded 3 times in a 6-hour exposure run.

Rectal temperature (RT)

The RT was taken twice a week before the SO_2 exposure using an electronic thermometer.

Radioimmunoassay

Serum cortisol concentrations in steers were determined by a single radioimmunoassay (RIA). The procedure was performed in duplicate using a solid phase DPC Coat-a-Count Kit (DPC TKCO2, Diagnostic Products Corporation, Los Angeles,

CA, USA) with the following modifications to a manufacture's protocol. To ensure that all sample potencies were estimated from the linear part of the standard curve the volume of serum sample for assay was increased from 25 µl to 125 µl, and the volume in the standards assay tubes was topped up to 125 µl with zero calibrator. An extra standard curve was generated by diluting 1:1 the lowest kit standard with zero calibrator, so the standards ranged from 0.25 to 12.5 ng/tube. Serial dilutions of standard bovine plasma showed parallelism to the standard curve. The assay sensitivity defined as 89.9% of total binding (TB) was 0.12 ng/tube. The recovery of a known amount of cortisol when added to steer sample of known potency was 95.2 %.

Metabolic rate

Energy metabolism expressed as heat production (HP) was recorded over an 18-hour period in experiment 1, and over 6 hours periods in experiments 2 and 3 using a respiration calorimetry system described by Young et al (1975) to assess the effects of SO_2 exposure on energy expenditure of the steers. The measurements were conducted towards the end of each SO_2 exposure period. The SO_2 treatments were discontinued on the day of the metabolic rate measurements.

The HP of the steers was calculated from the rate of oxygen (O_2) consumption obtained by respiration calorimetry using the formula developed by (McLean 1986): HP (Mcal day^{-1}) = 4.89 x O_2 (L/24 hr) / 1000; where, 4.89 is a constant representing heat/L of O_2 consumed and O_2 (L/24 hr) is the O_2 consumption per day. The calculations were performed by using a software calorimetry program "Westend WK4" (Dr. G. Mathison, Professor of the University of Alberta, Edmonton, AB). The metabolic rate was converted to Kcal d^{-1} expressed per metabolic body size: HP (Mcal d^{-1}) x $BW^{-0.75}$ x 1000, the units are Kcal d^{-1} $BW^{-0.75}$, where $BW^{-0.75}$ is the metabolic body weight. In experiments 2 and 3 the heat production output from the software was expressed on a 24-hour basis from the 6 hours of the respiration calorimetry procedure. The steers were fed in the hoods during the heat production measurements. The animals had good appetite and no feedbacks were recorded.

Before and after each calorimetry run, the oxygen analyzer was calibrated with 100% of nitrogen gas and a "span" gas consisted of 16.6% of oxygen, 2.06% of carbon

dioxide, 0.15% of methane and nitrogen to balance the mixture. To ensure quality and precision of the measurements, the validation of the entire calorimetry system was performed at the beginning of each experiment. The calibration procedure involved the infusion into the calorimetry system of known amounts of nitrogen to displace oxygen. By recovering the change in oxygen concentration during nitrogen infusion a mean value for oxygen recovery could be calculated. The mean and standard error of the oxygen recovery during the experiments was 99.1 ± 0.4%.

Statistical Analyses

Two steers in sequence were exposed to air with SO_2 in experiment 1, therefore, they both were designated as "treated" animals. The results across the exposure periods [pre-exposure (0ppm), 5 ppm, after 5 ppm, 20 ppm and after 20 ppm] are presented as arithmetic means of 2 steers. The steers in experiments 2 and 3 were grouped in blocks involving similar treatments, maintenance, and control conditions since only a few animals could be exposed to SO_2 simultaneously. The research study was performed using a factorial experimental design (treated versus control animals). The limitation of this study is that the same animals were exposed to various concentrations of SO_2 in sequence. We applied this experimental arrangement in order to reduce the total number of steers used in the project. For the purpose of statistical analysis, we considered animals exposed to 0 and 1 ppm, 0 and 5 ppm, 0 and 20 ppm of SO_2 as separate groups, and carry over effect of the treatments could not be determined. Data were analyzed using Proc GLM (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA) with fixed effect of treatments [SO_2 and control (no SO_2)], environment (warm and cold) where applicable, and two blocks. In experiments 2 and 3, the pre-exposure values for all dependent variables were included as a covariate. Metabolic rate was calculated as a change from initial pre-exposure values to control for individual animal differences, and the analysis of variance was performed on the changes from initial pre-exposure values for all groups of animals. The concentration of cortisol was analyzed by using repeated measures analysis of variance GLM procedure. Least Square Means (Lsmeans)

were estimated using the Pdiff option (SAS, Software Release 8.2, copyright 1999-2001, SAS Institute Inc., Cary, NC, USA).

RESULTS

Experiment 1

Clinical Observations

At the concentration of 5 ppm, the steers developed epiphora and increased mucous discharges from the nasal passages, an indication of the irritated mucous membranes. The steers appeared slightly depressed (lethargic and less responsive) after exposure to atmospheres with 5 ppm SO_2. At the 20 ppm exposure level, the steers appeared increasingly agitated, had epiphora, and enlarged blood vessels in the sclera. Within 3 days after SO_2 exposure, the animals developed a generalized increase in the inspiratory lung sounds and these sounds were more pronounced over the area of the diaphragmatic lobes.

All steers had blood parameters in a normal range, which are listed in Appendix B.

Heart rate, respiratory frequency, rectal temperature, body weight and feed consumption

The steers had heart rate (71-75 beats/min), rectal temperature (38.0-38.6°C) and respiratory frequency (27-30 breaths/min) in a normal range (Appendix A, Table A.1) and these parameters were not influenced by SO_2. Food and water consumption were not reduced by SO_2 exposure. The steers achieved the expected rate of gain (0.5 kg per day) for the level of feeding over the SO_2 exposure period (Table 7.1).

Cortisol

The mean concentrations of cortisol (6.3, 6.5 and 6.3 ng/ml) in blood serum of steers exposed to SO_2 were not different among the SO_2 exposure levels of 0, 5, and 20 ppm, respectively (Table 7.1).

Metabolic rate

The average initial metabolic rate (kcal/kg$^{-0.75}$/day) of the steers was 151, it was increased to 178 after exposure to 5 ppm of SO_2 (18%) and to 192 after exposure to 20 ppm of SO_2 (27%) (Table 7.1).

Experiment 2

Clinical observations

At the 1 ppm of SO_2 exposure level, changes were not observed in the skin or mucous membranes. Pulmonary sounds were normal. After exposure of animals to the 5 and 20 ppm levels the appearance of eyes, eyelids and lung sounds and behavior of the steers were similar to those that are described in experiment 1, however the clinical signs appeared on day 4 or 5 of exposure, and these observations were less pronounced than in experiment 1.

Body weight and feed consumption

No changes were observed in feed consumption and the animals gained weight (average 0.8 kg per day) over the period of SO_2 and control exposures (Table 7.2).

Heart rate (HR) and respiratory frequency (RF)

Mean values (69 – 79 beats/min) for heart rate (Table 7.3) and respiratory frequency (25 – 26 breaths/min) (Table 7.4) were in the normal range (Appendix A, Table A.1) and were not affected by SO_2 ($P > 0.05$).

Rectal temperature (RT)

Rectal temperature (T°C, Table 7.5) was in the normal range (Appendix A, Table A 1), however it was decreased by 0.1°C relative to controls during exposure to both 1 ppm level ($P< 0.10$) and by 0.2°C during 5 ppm of SO_2 exposure ($P< 0.01$). Rectal temperature was not affected by the 20 ppm ($P> 0.05$) level of SO_2 exposure.

Metabolic rate

During the pre-exposure period, mean metabolic rate (kcal/kg$^{-0.75}$/day) was similar for steers in the control (293) and SO_2 (275) groups (Table 7.6). The steers consumed the feed on offer with no refusals recorded on the days when metabolic rate was measured. Metabolic rate was increased by SO_2 exposure. The data are presented as a change from the pre-exposure values. Metabolic rate in control steers was significantly reduced ($P< 0.05$) during the subsequent experimental periods, especially those which corresponded to the periods the treated animals were exposed to atmospheres with 1 ppm (by 15%) and 5 ppm (by 3%) of SO_2. In the SO_2 treated animals there was no metabolic rate change at 1 ppm level ($P> 0.05$) but there were significant ($P< 0.05$) increases of 20% in metabolic rates at the 5 ppm and of 27% at 20 ppm levels of SO_2 exposure. The net result of these changes was that energy expenditure was significantly higher in SO_2 treated steers at 5 and 20 ppm levels ($P< 0.05$) then in control steers.

Experiment 3

Clinical observations

There were no visible changes on skin or mucous membranes in steers at 1 ppm of SO_2 exposure. Lung sounds were normal, animals behaved the same as they were in the pre-exposure period. After exposure of animals to the 5 and 20 ppm levels the appearance of eyes, eyelids and lung sounds and behavior of the steers were similar to those that are described in experiment 1, however the clinical signs appeared on day 4 or 5 of exposure, and these observations were less pronounced than in experiment 1.

Body weight and feed consumption

Feed and water refusal was not observed and all groups of animals gained approximately 0.8 kg/day or 30 kg of body weight over the series of exposure periods (Table 7.7).

Heart rate

Heart rate was reduced ($P< 0.05$) at 1 ppm of SO_2 in warm (by 7%) and in cold (by 8%) environments but not at the 5 or 20 ppm exposure levels of SO_2 (Table 7.8). Heart rate (73 – 77 beats/min) was not influenced by ambient temperature in the temperature controlled chamber ($P> 0.05$).

Respiratory frequency (RF)

Respiratory frequency (27 – 31 beats/min) (Table 7.9) in control steers and steers exposed to SO_2 were in the normal range (Appendix A, Table A.1) in both environments. However, RF in control steers was lower ($P < 0.05$) in the cold compared to the warm environment by 10% on average. Respiratory frequency was not affected ($P> 0.05$) by exposure to 1, 5 and 20 ppm of SO_2 in the cold environment (27 – 28 beats/min) compared to their corresponding controls, but RF was reduced by 10% ($P< 0.05$) at 5 ppm of SO_2 in the thermo-neutral environment compared to their control steers (Table 7.9).

Rectal temperature (RT)

Rectal temperature (38.4 – 38.7°C) (Table 7.10) was in a normal range (Appendix A, Table A 1). However, RT was decreased by 0.3°C during exposure to 5 ppm of SO_2 in the cold environment ($P< 0.05$), but was not affected by either 1 or 20 ppm ($P> 0.05$).

Cortisol

Neither environmental temperature ($P> 0.05$) nor exposure to atmospheres with SO_2 exposure ($P> 0.05$) affected the level of cortisol in blood serum of steers (Table 7.11). Also there were no differences ($P> 0.05$) in serum cortisol between blood sampling times within the exposure day across treatments.

Metabolic rate

Changing the ambient temperature without exposure to SO_2 did not significantly ($P> 0.05$) change metabolic rate. Metabolic rate was similar for all groups during the pre-exposure period (Table 7.12). Metabolic rate decreased in control animals during periods

corresponding to periods of 1, 5 and 20 ppm of SO_2, whereas, the metabolic rate increased in the SO_2 exposed steers. These data showed that metabolic rate was significantly increased ($P< 0.05$) by 33%, 39% and 44% at 1, 5 and 20 ppm of SO_2 when compared to control measurements in the cold environment but SO_2 exposure in the warm environment did not significantly change metabolic rate (temperature and treatment interaction, $P< 0.05$).

DISCUSSION

Exposure of the steers to atmospheres with 5 and 20 ppm SO_2 resulted in epiphora and seromucous discharge from the nostrils indicating irritation of mucous membranes. The changes in pulmonary sounds were also attributed to increased tracheal and bronchial secretions due to the effects of SO_2 on the respiratory epithelium. This observation is consistent with pulmonary cytology results that showed an increasing number of degenerative pulmonary cells isolated from bronchoalveolar lavage fluids obtained from these animals (see Chapter 5).

The epiphora was the result of irritation of the eye by SO_2 and a defensive response of increased tear production to rinse the eye. The enlarged blood vessels in the sclera are likely the result of vasoactive substances being release by inflammatory response and altering perfusion of the sclera. The crust observed around the eye is likely the result of increased mucous production (McClellan 1997). Our observations are supported by air pollution studies conducted in South Karelia, Finland (Jaakkola *et al* 1999, Marttila O 1994, Marttila *et al* 1994, Marttila *et al* 1995). These authors carried out epidemiological surveys among adults and children in order to establish acute health effects of malodorous sulfur compounds such as SO_2 and H_2S. Their results suggest that relatively low daily levels of malodorous sulfur compounds (in a range of 10 $\mu g/m^3$ or 0.004 ppm) cause exposure-related short-term adverse effects on eye, respiratory, and central nervous system symptoms. Similar increases in clinical symptoms of runny nose, teary eyes, and coughing were observed among the population of Hiroshima, Japan, from 1977-1992 and was associated with the increased average annual concentrations of SO_2 in ambient air (Setiani 1996). We observed more pronounced effects of SO_2 on the appearance of

mucous membranes and lung sounds in steers in experiment 1 compared to experiments 2 and 3. This finding could be due to the exposure of steers to 1 ppm of SO_2 prior to the inhalation of 5 and 20 ppm in the experiments 2 and 3, whereas experiment 1 was conducted only with the two higher concentrations of the air pollutant. It is possible that the prior exposure to 1 ppm allowed the animals in experiments 2 and 3 to develop some tolerance to SO_2.

Rectal temperature responses to SO_2 were not consistent. The decrease in rectal temperature during exposure to both 1 ppm and 5 ppm of SO_2 in experiment 2 and to 5 ppm in the cold environment of experiment 3 suggests some alterations in thermo-regulation, although no effects were observed at 20 ppm level of SO_2.

The changes in respiratory patterns and heart rate in steers over the duration of these experiments were not consistent. Heart rate was reduced only by 1 ppm level of SO_2 in both cold and warm environments in experiment 3. There was no indication from heart rate that the steers were stressed by inhalation exposure to SO_2. Respiratory rate was not affected by exposure to SO_2 in the cold environment but respiratory rate was reduced by exposure to SO_2 in the warm environment. It is known that humans and animals breathe more slowly and possibly with a larger tidal volume in the cold and tend to increase respiratory frequency and decrease tidal volume in warm environments. An altered breathing pattern helps homeothermic organisms to regulate their body temperature (e.g. panting in sheep and steers) (Foreman 1996). The cold environment, by decreasing respiratory frequency and possibly increasing tidal volume, may have masked an effect from breathing SO_2.

Respiratory rate and heart rate are often used as an indication of stress for an animal, and their alterations tend to be in direct proportion to heat production, although in some cases they can be dissociated from metabolic rate (Young 1975; McPhee *et al* 2003). Variable increases in energy expenditure of steers were observed at all levels of SO_2 exposure. These metabolic responses to SO_2 exposures were observed in the warm environment, as a trend in experiment 1 and significantly in experiment 2. It is not clear why metabolic rate was not significantly increased in experiment 3 in the warm environment, although a large increase occurred in response to SO_2 exposures in the cold, indicating an interaction of SO_2 and cold stress. The reason for the variations in response

is not certain, but might be due to the differences in age and background of animals in each experiment (Molnar and Schutz 1997). Also, the duration of SO_2 exposure periods prior to metabolic rate measurement was substantially longer in experiment 2 (28 days at 1 ppm, 14 days at 5 ppm and 9 days at 20 ppm) compared to experiment 3 (14 days at 1 ppm, 9 days at 5 ppm and 7 days at 20 ppm). Experiments 2 and 3 were conducted at different seasons (experiment 2 - in summer and experiment 3 - in fall/ winter), which might have influence on response to SO_2. Photoperiod is another variable that may affect metabolic rate (Boon 1997). Walker *et al* (1991) have shown that metabolic rate of sheep is suppressed in a declining photoperiod. Thus, metabolic responses to SO_2 might be reduced in the fall season.

Miaron *et al* (1995) reported increases of about 20% in heat production of yearling steers at -11°C compared to those at +10°C. Also, Hidari *et al* (1991) reported increases of approximately 8-10% in heat production of 2-year old steers at -20°C. Although energy expenditure is generally increased by cold stress, in this study there was no effect of temperature on the pre-exposure energy expenditure rates. The increase was only observed in the steers exposed to SO_2 in the cold temperature. The alteration of metabolic processes, such as increased protein synthesis and degradation in muscle tissue, improves survival of living organisms in a cold environment (Scott *et al* 1993), (Samuels *et al* 1996). Such may partly be the case for increased energy metabolism by steers housed in a cold environment.

Rectal temperature, which is often positively related to energy metabolism, was decreased at 5 ppm exposure level of SO_2 in the cold environment, at a time when energy metabolism was increased. Perhaps SO_2 influences the balance between heat production and heat loss to the environment. In our experiments, the highest level of SO_2 did not have an effect on rectal temperature. The reason for this effect is not known; however, the previous exposure of steers to 1 ppm level of SO_2 may have increased the tolerance to higher levels of SO_2. In addition, since the animals were exposed to SO_2 intermittently and only for 6 hours per day, it is possible that they were able to compensate during the rest periods between each exposure.

Plasma concentration of cortisol was not influenced by any level of SO_2 exposure or by environmental temperature. The influence of cold stress on level of cortisol in

ruminants is variable (Graham et al 1981). Lambs showed increased plasma cortisol concentration in cold environment exposure (Ekpe and Christopherson 2000), however serum cortisol concentrations were higher in heat-stressed cows compared to cows maintained in cool environment (Wise *et al* 1988). In the current study, the lack of increase in cortisol and heart rate suggest that the treatments did not induce severe or acute stress responses in the steers.

This study showed that exposure of cattle to SO_2 levels ranging from 1 to 20 ppm changed metabolic rate. Heat production was increased by SO_2 exposure in an exposure level-dependent manner. The increase in metabolic rate observed in response to SO_2 treatment indicated that the energy expenditure and, therefore, energy requirement of the animal exposed to SO_2 is increased. The energy expenditure increases were, on average, 10 – 15 % after intermittent exposure to 1 ppm SO_2, and 20-30% after exposure to 5 and 20 ppm SO_2 in the cold environment. These observations show that animals subjected to environmental SO_2 would require additional feed energy to compensate for the increased energy expenditure. The results indicate that, for example, for a 500 kg steer intermittently exposed to 1 ppm of SO_2 the extra feed required for maintenance and growth would be equivalent to an additional 1.5 kg day^{-1} of grain or 2.1 kg day^{-1} of hay (National Research Council (U.S.) and Subcommittee on Beef Cattle Nutrition 1996). It was not clear how long the increased energy expenditure would persist following the end of SO_2 exposure, but it appeared to last for at least 48 hours since metabolic rate measurements were conducted about 48 hours after the end of SO_2 exposure in experiment 1, and after 16 hours in experiments 2 and 3.

The cause of the higher metabolic rate is not known at the present time, but may represent part of a generalized stress response. The altered immune responses such as neutrophil respiratory burst (Chapter 3), rearrangement in quantity of peripheral blood lymphocyte subpopulations (Chapter 6), and evidence of inflammation of the pulmonary epithelium detected in steers inhaling SO_2 (Chapter 5) may be a part of the reason for the increased heat production. SO_2 exposure has been shown to increase bronchoconstriction and airway resistance in several species (Amdur 1974). If this also occurs in cattle, it could contribute to the higher metabolic rate by increasing the energy cost of breathing. Since the cattle were not actually exposed to SO_2 during the metabolic rate

measurements, the metabolic responses seen may have required a certain minimum duration of exposure to induce a gradual increase in resting metabolic rate. Such a delayed metabolic change is often observed in response to acclimation to other environmental factors, such as temperature, and may involve hormone alterations (Christopherson 2000). By analogy, a gradual metabolic acclimation response to SO_2 concentration and/or the immunological inflammatory changes associated with SO_2 may occur in cattle.

All animals had good appetites and ate all of the grain and alfalfa cubes on offer. The lack of effect of SO_2 exposure on feed intake might be attributed to the fact that the steers were not fed *ad libitum*. Assessment of effects of SO_2 on *ad libitum* feed intake would be of interest in view of the effect on energy metabolism, but was not an objective of the current study.

Table 7.1 Body weight, heart rate, body temperature, respiratory frequency and cortisol values of the steers exposed to 0, 5 and 20 ppm of SO_2, experiment 1

SO_2 treatment periods	Body weight (kg)	Heart rate (beats/min)	Rectal temperature (T^oC)	Respiratory frequency (breaths/min)	Cortisol[1] (µg/l)	Metrate (Kcal/ $kg^{-0.75}$
Pre-exposure (0 ppm)	644	72	38.5	28	25.2	151
5 ppm	649	71	38.3	27	26.0	178
After 5 ppm	657	73	38.6	30		
20 ppm	668	71	38.0	29	25.2	192
After 20 ppm	680	75	38.4	29		

1 – cortisol means averaged over the 5 hour sampling periods

Table 7.2 Mean body weight of control steers and steers exposed to 1, 5, and 20 ppm of SO_2, n = 6, experiment 2

Parameters	Body weight (kg) Lsmeans ± SEM	
Level of SO_2	Control[1]	SO_2
Pre-exposure	408 ± 7	385 ± 15
1 ppm	430 ± 17	403 ± 6
5 ppm	436 ± 8	449 ± 11
20 ppm	453 ± 9	470 ± 9

SEM = standard error of the mean
[1] control = atmospheric air

Table 7.3 Heart rate (beats/min) of control steers and steers exposed to various concentrations of SO_2, n = 6, experiment 2

	Treatment		
Level of SO_2	Control[1]	SO_2	SEM
Pre-exposure	79	81	2
1 ppm	76	74	1
5 ppm	69	72	2
20 ppm	70	72	1

SEM = pooled standard error of the mean
[1] control = atmospheric air

Table 7.4 Respiratory frequency (breaths/min) in control steers and steers exposed to 1, 5, and 20 ppm of SO_2, n = 6, experiment 2

	Treatment		
SO_2 Level	Control[1]	SO_2	SEM
Pre-exposure	26	25	1
1 ppm	26	25	1
5 ppm	25	26	1
20 ppm	25	25	1

SEM = pooled standard error
[1] control = atmospheric air

Table 7.5 Rectal Temperature (°C) in control steers and steers exposed to 1, 5 and 20 ppm of SO_2, n = 6, experiment 2

	Treatment		
SO_2 Level	Control[1]	SO_2	SEM
1 ppm	38.8	38.7^{+}	0.1
5 ppm	38.7	38.5**	0.1
20 ppm	38.8	38.7	0.1

+ ($P< 0.10$) ** ($P<0.01$)
SE = pooled standard error;
[1] control = atmospheric air

Table 7.6 The pre-exposure metabolic rate and changes in metabolic rate from initial pre-exposure values (kcal $kg^{-0.75}d^{-1}$) of control steers and steers exposed to 1, 5, and 20 ppm of SO_2, n = 6, experiment 2

		Treatment		
		Control	SO_2	SEM
Pre-exposure Metabolic rate (kcal $kg^{-0.75}$ /day)		293	275	37
Variables	Level of SO_2			
Change	1	-21.5	-1.42	9.8
Change	5	-44.6^{b}	12.64^{a}	18.2
Change	20	-9.2^{b}	67.4^{a}	18.7

a, b = Means within a row with different superscripts are different ($P< 0.05$)
SEM = Standard error of the mean
Control = atmospheric air

Table 7.7 Body weight (kg) of control steers and steers exposed to 1, 5, and 20 ppm of SO_2 in warm and cold environments), experiment 3, n = 4, (Lsmeans ± SEM)

Environment	Warm		Cold	
Level of SO_2	Control[1]	SO_2	Control[1]	SO_2
1 ppm	541 ± 26	528 ± 14	551 ± 82	542 ± 29
5 ppm	556 ± 24	548 ± 13	560 ± 29	537 ± 28
20 ppm	574 ± 27	562 ± 14	579 ± 77	570 ± 21

SEM = standard error of the mean
[1] control = atmospheric air

Table 7.8 Heart rate (beats/min) of control steers and steers exposed to 1, 5, and 20 ppm of SO_2 in warm and cold environments, experiment 3, n = 4

	Treatment				
	Warm		Cold		SEM
Level of SO_2	Control[1]	SO_2	Control[1]	SO_2	
1 ppm	83^{a}	77^{b}	83^{a}	76^{b}	1
5 ppm	75	73	76	75	2
20 ppm	74^{ab}	77^{a}	76^{ab}	73^{b}	2

a, b, means within a row with different superscripts differ ($P < 0.05$).
SEM = pooled standard error;
[1] control = atmospheric air

Table 7.9 Respiratory frequency (breaths/min) in control steers and steers exposed to 1, 5, and 20 ppm of SO_2 in warm and cold environments. Experiment 3, n = 4

	Treatment				
	Warm		Cold		
Level of SO_2	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	30^{a}	29^{ab}	28^{b}	27^{b}	1
5 ppm	31^{a}	28^{b}	26^{b}	27^{b}	1
20 ppm	29^{ab}	31^{a}	27^{b}	28^{b}	1

ab, means within a row with different superscripts differ ($P < 0.05$)
SEM = pooled standard error of the mean
[1] control = atmospheric air

Table 7.10 Rectal temperature (°C) in control steers and steers exposed to 1, 5, and 20 ppm of SO_2 in warm and cold environments. Experiment two, n = 4

	Treatment				
SO_2 Level	Warm		Cold		
	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	38.5	38.5	38.5	38.6	0.1
5 ppm	38.6^{ab}	38.5^{b}	38.7^{a}	38.4^{b}	0.1
20 ppm	38.5	38.5	38.5	38.6	0.1

a, b = Means within a row with different superscripts are different ($P < 0.05$)
SEM = pooled standard error of the mean
[1] control = atmospheric air

Table 7.11 Concentrations of cortisol (µg/l) in serum of steers exposed to 0, 1, 5 and 20 ppm of SO_2 in warm and cold environments. Experiment 3, n = 4

	Treatments				
Level of SO_2	Warm		Cold		
Cortisol (ng/ml)	Control[1]	SO_2	Control[1]	SO_2	SEM
1 ppm	19.2	32.0	25.6	25.6	0.6
5 ppm	23.2	20.8	23.2	23.2	0.1
20 ppm	18.4	40.0	17.6	15.2	0.2

SEM = standard error of the mean
[1] control = atmospheric air

Table 7.12 Initial heat production and change in heat production during treatment for control steers and steers exposed to 1, 5, and 20 ppm SO_2 in warm and cold environments, n = 4, experiment 3.

	Environment	Warm		Cold		SEM
	SO_2 level	Control[1]	SO_2	Control[1]	SO_2	
Initial HP ($kcalW^{-0.75}$/day)	0 ppm	278	259	291	279	23.9
Change in HP ($kcalW^{-0.75}$/day)	1 ppm	35^{ab}	17^{ab}	-26^{b}	67^{a}	17
Change in HP ($kcalW^{-0.75}$/day)	5 ppm	3.3^{ab}	-1.5^{ab}	-55.7^{b}	55.5^{a}	24.8
Change in HP ($kcalW^{-0.75}$/day)	20 ppm	9.1^{ab}	-1.1^{ab}	-54.4^{b}	72.0^{a}	21.0

ab means with different superscripts within a row differ ($P < 0.05$)
SEM = pooled standard error of the mean
[1] control = atmospheric air

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Chapter 8

GENERAL SUMMARY AND DISCUSSION

SUMMARY of major findings:

Chapter 2 describes the small but significant variation in SO_2 levels among the SO_2 exposure hoods. As a result we rotated the animals between the hoods to prevent a possibility of uneven exposure of different steers to the SO_2 gas.

Chapter 3 presents evidence that the stress from inhalation of air with SO_2 had a negative influence on blood neutrophils, which are the first line of immune defense against infection. The oxidative rate of the blood neutrophils was decreased in steers at all levels of SO_2 exposure in thermo-neutral environment suggesting the possibility that these animals could have an impaired ability to cope with disease. We were not able to test this assumption in the current study. Future investigations will be required to assess disease resistance of cattle exposed to SO_2.

Chapter 4 provides evidence for a decreased vitamin E concentration in the blood plasma of the steers exposed to air with SO_2 as a possible consequence of combating oxidative stress caused by the gas exposure. However, unaltered concentrations of acute phase proteins in blood serum of steers indicated that the stress from the SO_2 inhalation was not strong enough to cause a major systemic inflammatory response in the animals.

Chapter 5 presents evidence that direct exposure of the respiratory system to the air with SO_2 had deleterious effect on the pulmonary cells in steers. The analysis of the bronchoalveolar lavage (BAL) fluids collected from the steers demonstrated an increased ratio of degenerative to normal pulmonary cells. Also an increased activity of lactate dehydrogenase and alkaline phosphatase in BAL fluids support the evidence about the occurrence of at least mild inflammatory processes in animals' lungs in response to SO_2 exposure. We also present evidence that NO production by the pulmonary macrophages was decreased, which could limit the animal's immune defense against infection or other causative inflammatory agents. In addition, we demonstrated that cold stress and SO_2 possibly have interactive effects in that the production of NO by the pulmonary macrophages was more suppressed in animals exposed to a cold environment, compared

to the animals in a warm environment. However, we are not able to specify if this was an additive or synergistic effect of the interaction. This question requires further research and could be answered with focused future toxicological studies.

Chapter 6 presents evidence that inhalation of air with SO_2 at 1, 5 and 20 ppm was strong enough to induce some effects on mononuclear leukocytes of the immune system, in steers, even though the stress was not strong enough to cause generalized inflammatory disease in animals. It was found that the ratio of CD4 (helper T cells) to CD8 (cytotoxic T cells) was decreased, suggestive of an altered balance of immuno-regulatory cytokines released from the pulmonary system of steers as a result of local cell damage. We speculate that the immune cell responses in the animals exposed to SO_2 could have a negative effect on immunity. However, the ultimate impact on future health and disease resistance of the steers is uncertain. Also, we were not able to specify what cytokines were participating in this response. These are areas that require further investigations.

Chapter 7 presents evidence for increased metabolic rate in steers in response to exposure to 5 and 20 ppm of SO_2 and that metabolic rate was exacerbated by the cold stress. Effects of SO_2 on heart rate, rectal temperature, and concentration of cortisol, which are often considered as indicators of animal stress, were also examined. This chapter also summarizes observations on visible clinical signs (increased lung sounds, appearance or mucous membranes, and behavior) in steers, responding to SO_2 treatment, however these observations are subjective.

DISCUSSION

It is important to note that, although we exposed cattle to 1, 5 or 20 ppm of SO_2, the exposure times were of limited duration (6 h per day for 5 out of every 7 days) amounting to 17.5% of the time. Therefore long term average exposure doses in these studies would be equivalent to 0.175, 0.575 and 3.5 ppm on a continuous basis, instead of 1, 5 and 20 ppm. This must be kept in mind when considering the implications of the results. The major outcomes of the studies involving SO_2 exposure were expected to include impacts on the pulmonary system in view of previous reports of the effect of SO_2 on respiratory parameters in humans and laboratory animals (Amdur 1985). To our

knowledge, this study represents the first report on controlled studies on SO_2 exposure in live cattle, although some in vitro studies have previously examined the effect of sulfite on bovine neutrophils (Komarnisky *et al* 2000) exposed under incubation *(in vitro)* conditions. However, effects of SO_2 exposure on the pulmonary immune or general immune defense system in humans and animals have not been previously reported. The present study appeared to be the first to document integrated measurements of metabolic rate, blood neutrophil respiratory activity, blood lymphocyte subtypes redistribution and production of NO by pulmonary macrophages in response to SO_2 exposure, not only in cattle but in humans or any animal species. Studies on the effect of SO_2 inhalation on the status of pulmonary epithelial cells were performed in rats (Krasnowska *et al* 1998), however, we did not find any reports documenting changes in differential pulmonary cell count in any species in response to SO_2.

We report on the impairment of the components of the immune system and the defense mechanism of the pulmonary system by the SO_2 exposure at various levels. It appeared that SO_2 exposure induces some responses in the animal in a concentration-dependent manner, and other responses may have been threshold in nature, such as marginal effect at the exposure steers to 1 or 5 ppm of SO_2 (e.g. trend to increase energy metabolism). Future investigations may help to clarify whether there are linear or threshold effects in relation to SO_2 on the body functions of the steers. Due to individual animal variability, and as a result of a small number of degrees of freedom (limited number of animals in each study) the power of statistical tests is only 60% in some cases, which means that the experiment design may have failed to find the real difference (should there have been the difference) 4 times out of 10. The sample size calculations (Chapter 5, page 135) demonstrate that the trend to decrease NO production by pulmonary macrophages ($P=0.06$) at 5 ppm of SO_2 exposure might have been significant ($P< 0.05$) with the use of 8 steers per treatment in the experiment. However, the results obtained indicate that the animals did appear to be at risk during exposure to 5 ppm of SO_2, which is the level that humans may be exposed in the working environments for 15 minutes a day (ACGIH 1996). This study is the first to show that the immune system responds to environmental pollution (SO_2), and is affected by the pollutant at levels, which produced only mild clinical signs of intoxication or even in the absence of clinical

signs. Future investigations should focus on determining the mechanism by which SO_2 affects the immune system.

Sulfur dioxide hydrates in biological fluids to sulfuric acid with a subsequent dissociation to bisulfites, sulfites and hydrogen ions (Hippeli et al 1995). Sulfites participate in synthesis of sulfur amino acids and other endogenous sulfur containing compounds, as well as interacting with buffers in the biological fluids when they are present in amounts tolerated by the body. However, increased amounts of sulfur moieties may directly participate in the production of free radicals (Hippeli et al 1995), or indirectly as a highly reactive non-metal, which may be conjugated with microelements, required in production of vitamins, and antioxidative enzymes (glutathione and superoxide dismutase). This is supported by *in vitro* studies with red blood cells which showed that lipid peroxidation processes in cell membranes and decreases in oxidative enzymes activity (Gumuslu *at al* 1998) resulted in response to SO_2 exposure. This may be a possible mechanism for influences on the function of immune cells. Another mechanism may be related to the increased release of cytokines from the damaged cells as a result of the direct action of protons (H^+) released after hydration of SO_2 in the biological fluids.

We suggest that cytokines produced by increased numbers of damaged pulmonary cells could be possible mediators passing signals to the immune network to recruit neutrophils and macrophages to clean the cell debris. This is in agreement with the increased number of blood monocytes and $CD8^+$ (cytotoxic T cells) cells as found in our study. It is possible that the immune system needs more monocytes to compensate for the damaged alveolar macrophages or the macrophages which may have lost their phagocytic activity (decreased production of NO, as found in this study) and decreased chemotactic function as was found in the *vitro* study with alveolar macrophages directly exposed to SO_2 (Knorst *et al* 1996). The decreased number of $CD4^+$ cells in blood observed in these experiments also may be related to the decreased production of NO, as a previous study (Liew 1995) found that cytokines TNF and IL-4 produced by T helper cells are participating in the regulation of NO production by alveolar macrophages. The decreased ratio of $CD4^+$ to $CD8^+$ cells observed in blood of steers exposed to SO_2 suggests the possibility of immunological impairment due to decreased T helper cells and increased T-

suppressor cells. On the other hand, the higher CD8 cell number could also be an indicator of a shift from humoral to cell-mediated immunity. It is possible that cytotoxic effect of SO_2 on the tissues of the pulmonary system is responsible for the differentiation of lymphocytes into cytotoxic/suppressor T cells because there were no alterations in total white blood cell numbers of steers due to exposure to SO_2. The health consequences of such a shift are uncertain. During inhalation of SO_2, inflammation or cytotoxicity could be expected in the tissues of the pulmonary system, and leukocyte trafficking into pulmonary tissue would likely also occur as a component of the host defense response (Wagner and Roth 2000).

NO is synthesized endogenously by nitric oxide synthase (NOS) isoenzymes that oxidize l-arginine to l-citrulline and are present in a variety of cell types, including vascular endothelial cells, smooth muscle cells, platelets, neuronal cells, macrophages, and neutrophils (Moncada 1991). There are 3 forms of NOS that have been described: constitutive NOS (neuronal NOS [type I], endothelial NOS [type III]) isoforms and an inducible NOS (iNOS, type II). NO is a small lipophilic molecule with a biological half-life of several seconds; thus, it can easily diffuse into and across plasma membranes and lipoproteins. As a free radical, NO readily reacts with other radical species such as superoxide, lipid alkoxyl, and peroxyl radicals and the metal centers of metalloproteins (Mayer 1997). The iNOS is upregulated by cytokines as well as bacterial cell wall components (Yoo 1996). Our steers were free from infection at the time of the experiment; therefore, we may conclude that *in vivo* the enzyme was stimulated by mediators from the damaged pulmonary cells. We do not know the rate of NO production *in vivo*, however *in vitro* it was decreased. It is possible, that SO_2 exposure limited the substrate (L-arginine) or damaged the enzyme itself, or interfered with a component of the NO oxide production pathway.

In addition to our speculation that NO production by alveolar macrophages and neutrophil respiratory burst was also inhibited by the action of SO_2, we may stipulate another mechanism. Nitric oxide has been shown to regulate inflammatory responses that are relevant to airway defense mechanism such as neutrophil adhesion and chemotaxis (Moncada and Higgs 1993). Superoxide anion is continuously inactivated by NO under normal conditions (Liew 1995). Sulfur dioxide rapidly dissociates in the moisture of

mucous membranes and in biological fluids, producing sulfites and bisulfites, which are representatives of free radicals. Through the process of lipid peroxidation which may occur in the cell membrane due to the action of these radicals (Meng 2003) the pathway for nitric oxide synthesis may be disrupted. Nitric oxide is also known as a protective agent by scavenging superoxide directly, as mentioned above, or indirectly, by inhibiting neutrophil superoxide production ((Liew 1995).

In the present study, the steers appeared to be free from any pathogenic infections. The increase observed for the proportion of $CD25^+$ cells ($P< 0.01$) is indicative of interleukin-2 receptor. Since the IL-2 cytokine normally signals the immune system to increase the production of $CD4^+$ lymphocytes (Ayoub and Yang 1995), an increase in IL-2 receptor expression may prepare the system to eventually proliferate more lymphocytes into helper T cells. The decreased percentage of $TcR1^+$ (T-cell receptor) ($P< 0.05$) is consistent with the decrease actually observed in the $CD4^+$ lymphocyte population expressing alpha/beta TcR1 surface antigen receptors (Clevers et al 1990).The results of this study indicate that the levels of SO_2 used in the experiments produced a cytotoxic effect on the pulmonary system, rather than broad generalized inflammation. This is in agreement with our findings, such as increased LDH and ALP in the BAL fluids (indicators of the cellular damaged) and at the same time absence of increase in circulating acute phase proteins, which are the indicators of generalized inflammations. It is not known what mechanism is responsible for the cytotoxic effect of SO_2 on the lung tissue, however we cannot rule out the possibility of oxidative injury to the cells (Hippeli *et al* 1997). The increased number of cytotoxic T cells and monocytes observed in our study may indicate that there is an increased need for phagocytic and cell-mediated functions of immune defense. However, future focused immunological studies will be needed to clarify this phenomenon.

The increased lung inspiratory sounds and increased ratio of degenerative to healthy pulmonary cells detected in the steers exposed to SO_2 (Chapters 7 and 5), may indicate an initiation of mild localized inflammatory processes or cytotoxicity due to exposure over 4-5 days at 5 ppm of SO_2 followed by subsequent increase to 20 ppm level of air with SO_2. However, it is not known how long such an inflammatory process or cell damage will persist if exposure to air with SO_2 continues.

Increased metabolic rates at the whole animal level indicate that possible changes in cellular metabolic activity were induced with SO_2 exposure, and this may also be related to the function of immune cells. It is not clear exactly what mechanisms might be inducing generalized increases in metabolic rate or what tissues/organ systems contributed to the response. It is possible that some of the mediators arising from the lung inflammation or cytotoxicity could induce changes in metabolism of the steers as well as changes in immune cell function. Baracos *et al* (1987) have shown that LPS in sheep induces a large metabolic response in association with a febrile response. Since we did not observe significant increases in acute phase proteins in response to SO_2 in our steers, a smaller metabolic change than that observed by Baracos *et al* (1987) would also be anticipated. Indeed, the steers in our experiments did not show a febrile response, and their metabolic rate changes were much smaller than the response reported by Baracos *et al* (1987). Other studies have shown that a variety of injuries can induce respondent increases in metabolic whole body energy expenditure due to mediators of inflammation and tissue damage (Chiolero 1997). It may be that a pattern of cytokines released in response to localized pulmonary damage could induce moderate, but significant metabolic changes. Further research is needed to determine the mechanisms responsible for both the immunological and metabolic changes. Although the mechanisms leading to increased metabolic rate have not been identified, the consequences of these changes for the energy requirements of the steers would appear to be substantial. For example, if the increase in energy expenditure will be, on average, 10 – 15% after exposure to air with SO_2, this amount of additional feed energy would be required to compensate for SO_2 exposure. For a 500 kg steer, the extra feed required for maintenance and growth could be supplied by approximately 1.5 kg/day of grain or 2.1 kg/day of hay (National Research Council 1996).

The in vitro studies of the intracellular effects of sulfite on human (Beck-Speier *et al* 1994) and bovine (Komarnisky *et al* 2000) neutrophils support our in vivo findings that SO_2 suppresses the neutrophil respiratory burst activity. In the cold environment, the suppression of neutrophil activation was limited to the 20-ppm exposure level, suggesting a moderating effect of cold exposure for this aspect of immunological function. However, there was no evidence that cold exposure enhanced neutrophil activation in control steers.

In conclusion, the results presented in this thesis provide convincing evidence that the exposure of cattle to air with SO_2 concentrations ranging from 1 to 20 ppm affects cells of the immune and pulmonary systems. Fewer deleterious effects were observed in cattle at 1 ppm of SO_2, although there was evidence of altered immune function even at this lower level of SO_2. The immune cells did continue to respond to stimulation *in vitro* (neutrophils by PMA and macrophages by LPS) in steers exposed to SO_2, however there was a significant suppression of neutrophil respiratory activity and macrophage NO production by SO_2. There was also evidence for increased maintenance energy requirements of the steers by 10 to 15 % as a result of SO_2 exposure.

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APPENDIX A

Table A.1 Normal values of physiological parameters for adult beef cattle (Reece 1997)

Prameters	Units	Range	Average
Heart rate	Beats/min	60 – 80	70
Rectal temperature	T°C	36.7 – 39.7	38.3
Respiratory frequency	Breath/min	10 – 30	20

Reece WO (1997), *Physiology of domestic animals*, 2nd ed, Williams&Wlkons, A Waverly Company, Baltimore, MD, USA

APPENDIX B

The abbreviations of the hematological parameters

Parameters	Column Abbreviations	Units
Red Blood Cell Count	RBC	x 10^{12}/L
Hemoglobin	HGB	g/L
Hematocrit	HCT	L/L
Mean Cell Volume	MCV	fL
Mean Cell Hemoglobin	MCH	pg
Mean Cell Hemoglobin Concentration	MCHC	g/L
Platelet	PLT	x 10^{9}/L
White Blood Cell Count	WBC	x 10^{9}/L
Segmented Neutrophil	SEG	
Eosinophil	EOS	
Basophil	BASO	
Lymphocyte	LYMPH	
Monocyte	MONOS	
Absolute Segmented Neutrophil Count	ABSSEG	x 10^{9}/L
Absolute Eosinophil Count	ABSEOS	x 10^{9}/L
Absolute Basophil Count	ABSBASO	x 10^{9}/L

Table B.1 Hematological values of peripheral blood from steers exposed to 0, 1, 5 and 20 ppm of SO_2 (Experiment 2)

SO_2 treatment	Hb		PCV		RBC		MCV	
Reference	80-150		0.24-0.46		5-12		40 - 60	
Units	g/L		L/L		tera/L		fL	
	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	124.4	13.9	0.3	0.04	8.6	0.8	38.1	2.9
Pre-exposure	131.2	10.7	0.3	0.03	8.9	0.8	39	4.5
Control 1 ppm	122.3	13.8	0.3	0.03	8.2	0.8	40.3	3.2
1 ppm	129.1	9.6	0.4	0.03	8.6	0.9	40.6	4.4
Control 5 ppm	122.6	15.1	0.3	0.04	8	0.9	41.6	3.6
5 ppm	126.4	11.6	0.3	0.03	8.2	0.9	41.8	4.8
Control 20 ppm	123	16.6	0.3	0.04	7.8	1	42.9	3.5
20 ppm	130.2	11.3	0.4	0.03	8.3	0.9	42.9	4.9

SO_2 treatment	MCH		MCHC		WBC	
Reference	11-17		300-360		4000-12000	
Units	pg		g/L		/mm^3	
	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	14.4	1	378.8	5.9	9304.2	1532.8
Pre-exposure	14.7	1.3	377.8	10.1	9146.7	1687.8
Control 1 ppm	14.9	1.1	369.6	5.8	8694	1581.3
1 ppm	15	1.4	370.2	10.3	9467.3	1949
Control 5 ppm	15.4	1.2	370.4	5.7	8792.2	1600.5
5 ppm	15.6	1.6	372.1	10.2	9546.7	1843.7
Control 20 ppm	15.6	1.4	368	2.2	9650	854.9
20 ppm	15.9	1.4	367.8	9.6	9483.3	1915.6

Table B.1 (Cont'd) Hematological values of peripheral blood from steers exposed to 0, 1, 5 and 20 ppm of SO_2 (Experiment 2)

SO_2 treatment	Lymphocytes				Monocytes			
	count		%		count		%	
Reference	2500-7500				25-840			
Units	/mm^3				/mm^3			
	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	6212.2	987.9	67.3	7.9	393.4	312.7	4.01	2.9
Pre-exposure	5878.2	1903.9	63.8	12.1	303.5	154.7	3.4	1.6
Control 1 ppm	5834.9	1365.9	66.9	7.7	420	300.7	4.8	3.4
1 ppm	6081.6	2059.8	63.9	14.2	595.2	1126.6	6	10
Control 5 ppm	6103.1	1068.6	69.6	6.2	345.4	255.5	3.9	2.8
5 ppm	6669.2	1760.3	69	8.2	368.1	174	3.8	1.7
Control 20 ppm	6818.5	1028.4	70.8	10.5	140.8	64.2	1.4	0.5
20 ppm	6942	1824.5	72.7	6.4	499.2	268.1	5.2	2.5

SO_2 treatment	Neuts				Bands	
	count		%		count	%
Reference	2500-7500				0-120	
Units	/mm^3				/mm^3	
	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	2387.2	973.4	25	8.3	82	
Pre-exposure	2823.5	945.8	32	12.8		
Control 1 ppm	2111.8	782.1	25	9.5	32.5	44.5
1 ppm	2489.8	625.1	27	7.1		
Control 5 ppm	2093.6	755.7	23.1	6.1	84	
5 ppm	2261.6	724.4	24.6	9.7		
Control 20 ppm	1925	799.9	19.8	7.8		
20 ppm	1937.3	477.4	20.8	5.8		

SO_2 treatment	Eosinophils				Basophils			
	count		%		count		%	
Reference	0-2400				0-200			
Units	/mm^3				/mm^3			
	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	351.2	225.9	4.2	2.7	93	16.9	1	
Pre-exposure	325.1	245.4	2.5	1.4				
Control 1 ppm	346.6	368.3	4.6	4.1				
1 ppm	313.7	281.9	3.1	2.7	228.8	186.9	2.4	1.9
Control 5 ppm	207.1	195.2	4	2.8	83	20.2		
5 ppm	314.9	288.3	3.2		107		1	
Control 20 ppm	904.8	1405.5	9.4	14.5	105.5	0.7	1	0
20 ppm	271.2	253.6	3	3.1				

Table B.2 Hematological values of peripheral blood from steers exposed to 0 and 1 ppm of SO_2 (Experiment 3)

		RBC		HGB		HCT		MCV	
Reference	low	6.05		99		0.27		34	
Reference	high	9.70		156		0.44		56	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure Control	warm	8.11	0.92	125	13	0.33	0.03	41	3
Pre-exposure	warm	8.58	0.84	132	9	0.35	0.02	41	4
Pre-exposure Control	cold	8.65	0.78	131	9	0.35	0.02	41	6
Pre-exposure	cold	7.48	1.09	128	20	0.35	0.06	46	4
1 ppm Control	warm	8.01	0.89	121	11	0.21	0.03	40	3
1 ppm	warm	8.47	0.92	130	8	0.33	0.02	40	4
1 ppm Control	cold	8.81	0.75	132	13	0.35	0.03	40	5
1 ppm	cold	7.73	0.50	134	15	0.36	0.04	47	3

		MCH		MCHC		PLT		WBC	
Reference	low	13		329		189		4.5	
Reference	high	19		382		662		12.5	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure Control	warm	15	1	373	5	391	134	9.8	3.1
Pre-exposure	warm	15	1	375	7	385	80	9.8	1.7
Pre-exposure Control	cold	15	2	376	7	422	47	8.2	1.7
Pre-exposure	cold	17	1	371	8	393	126	9.0	2.0
1 ppm Control	warm	16	1	369	7	400	45	8.1	1.5
1 ppm	warm	17	1	369	7	389	124	8.7	1.9
1 ppm Control	cold	15	2	375	11	401	52	8.4	1.8
1 ppm	cold	17	1	369	8	421	47	9.8	1.9
1 ppm Control	cold	15	2	375	11	401	52	8.4	1.8
1 ppm	cold	17	1	369	8	421	47	9.8	1.9

Table B.2 (Cont'd) Hematological values of peripheral blood from steers exposed to 0 and 1 ppm of SO_2 (Experiment 3)

		SEG		ABSSEG		LYMPH		ABSLYMPH	
Reference	low	0.05		0.22		0.48		2.20	
Reference	high	0.37		4.59		0.93		11.61	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure Control	warm	0.19	0.09	0.61	0.66	0.70	0.08	6.89	2.30
Pre-exposure	warm	0.19	0.05	0.72	0.78	0.72	0.07	7.00	0.77
Pre-exposure Control	cold	0.17	0.04	0.72	0.85	0.73	0.07	5.97	1.06
Pre-exposure	cold	0.17	0.02	0.79	0.89	0.76	0.03	6.79	1.37
1 ppm Control	warm	0.18	0.08	0.59	0.58	0.70	0.08	6.79	2.27
1 ppm	warm								
1 ppm Control	cold	0.21	0.04	1.13	0.96	0.69	0.06	5.71	1.22
1 ppm	cold	0.21	0.03	1.45	1.13	0.72	0.03	7.07	1.30

		MONOS		ABSMONO		EOS		ABSEOS	
Reference	low	0.00		0.00		0.00		0.00	
Reference	high	0.08		1.03		0.14		1.77	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure Control	warm	0.02	0.01	0.13	0.01	0.09	0.02	0.87	0.46
Pre-exposure	warm	0.02	0.01	0.14	0.05	0.07	0.04	0.70	0.51
Pre-exposure Control	cold	0.02	0.01	0.13	0.07	0.08	0.03	0.64	0.31
Pre-exposure	cold	0.02	0.01	0.15	0.10	0.06	0.04	0.51	0.50
1 ppm Control	warm	0.02	0.01	0.12	0.01	0.08	0.02	0.85	0.29
1 ppm	warm	0.02	0.01	0.11	0.01	0.06	0.04	0.71	0.49
1 ppm Control	cold	0.02	0.01	0.15	0.05	0.09	0.03	0.72	0.31
1 ppm	cold	0.01	0.01	0.13	0.06	0.06	0.03	0.57	0.34

Table B.2 (Cont'd) Hematological values of peripheral blood from steers exposed to 0 and 1 ppm of SO_2 (Experiment 3)

		BASO		ABSBASO		FIBRINOGEN	
Reference	low	0.00		0.00		0.53	
Reference	high	0.01		0.08		1.53	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure Control	warm	0.01	0.01	0.04	0.04	2.50	1.00
Pre-exposure	warm	0.01	0.01	0.07	0.05	2.50	0.58
Pre-exposure Control	cold	0.01	0.01	0.04	0.04	2.75	0.96
Pre-exposure	cold	0.01	0.01	0.06	0.07	2.25	0.50
1 ppm Control	warm	0.01	0.01	0.05	0.04	0.61	0.78
1 ppm	warm	0.0.1	0.01	0.04	0.04	2.47	0.61
1 ppm Control	cold	0.01	0.01	0.04	0.05	2.00	0.89
1 ppm	cold	0.00	0.00	0.02	0.04	1.83	0.41

Table B.3 Hematological values of peripheral blood from steers exposed to 5 ppm of SO_2 (Experiment 3)

		RBC		HGB		HCT		MCV	
Reference	low	6.05		99		0.27		34	
Reference	high	9.70		156		0.44		56	
SO_2 treatment	Environment	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
5 ppm Control	warm	8.64	8.50	123	126	0.34	0.34	39	40
5 ppm	warm	8.52	0.39	121	6	0.33	0.02	39	0
5 ppm Control	cold	8.89	0.21	130	17	0.35	0.05	39	5
5 ppm	cold	7.85	8.01	147	141	0.39	0.38	50	48

		MCH		MCHC		PLT		WBC	
Reference	low	13		329		189		4.5	
Reference	high	19		382		662		12.5	
SO_2 treatment	Environment	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
5 ppm Control	warm	14	15	365	373	356	458	12.9	10.7
5 ppm	warm	14	0	368	3	345	49	9.6	0.8
5 ppm Control	cold	15	2	376	6	345	16	8.3	2.9
5 ppm	cold	19	18	376	368	368	377	10.2	9.7

		SEG		ABSSEG		LYMPH		ABSLYMPH	
Reference	low	0.05		0.22		0.48		2.20	
Reference	high	0.37		4.59		0.93		11.61	
SO_2 treatment	Environment	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
5 ppm Control	warm	0.17	0.17	2.19	1.76	0.76	0.72	9.80	7.72
5 ppm	warm	0.22	0.05	2.07	0.41	0.68	0.05	6.54	0.87
5 ppm Control	cold	0.21	0.03	1.72	0.65	0.68	0.02	5.65	1.93
5 ppm	cold	0.23	0.20	2.35	1.93	0.74	0.73	7.55	7.06

Table B.3 (Cont'd) Hematological values of peripheral blood from steers exposed to 5 ppm of SO_2 (Experiment 3)

		MONOS		ABSMONO		EOS		ABSEOS	
Reference	low	0.00		0.00		0.00		0.00	
Reference	high	0.08		1.03		0.14		1.77	
SO_2 treatment	Environment	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
5 ppm Control	warm	0.01	0.02	0.13	0.18	0.06	0.09	0.77	0.98
5 ppm	warm	0.02	0.01	0.15	0.06	0.09	0.02	0.84	0.19
5 ppm Control	cold	0.02	0.01	0.18	0.05	0.08	0.02	0.70	0.32
5 ppm	cold	0.00	0.02	0.00	0.17	0.03	0.06	0.31	0.56

		BASO		ABSBASO		FIBRINOGEN			
Reference	low	0.00		0.00		0.53			
Reference	high	0.01		0.08		1.53			
SO_2 treatment	Environment	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
5 ppm Control	warm	0.00	0.00	0.00	0.03	2.00	2.50		
5 ppm	warm	0.00	0.00	0.00	0.00	2.00	0.00		
5 ppm Control	cold	0.01	0.01	0.06	0.05	1.75	0.50		
5 ppm	cold	0.00	0.00	0.00	0.02	1.00	1.50		

Table B.4 Hematological values of peripheral blood from steers exposed to 20 ppm of SO_2 (Experiment 3)

		RBC		HGB		HCT		MCV	
Reference	low	6.05		99		0.27		34	
Reference	high	9.70		156		0.44		56	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm Control	warm	8.50	8.47	123	128	0.33	0.34	38	40
20 ppm	warm	9.07	0.75	131	11	0.36	0.03	39	0
20 ppm Control	cold	9.35	0.14	137	15	0.36	0.04	39	5
20 ppm	cold	7.65	8.24	146	147	0.38	0.39	50	48

		MCH		MCHC		PLT		WBC	
Reference	low	13		329		189		4.5	
Reference	high	19		382		662		12.5	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm Control	warm	14	15	377	377	344	458	11.2	9.5
20 ppm	warm	15	0	367	5	329	52	9.7	0.8
20 ppm Control	cold	14	2	377	6	340	30	9.9	2.7
20 ppm	cold	19	18	381	374	319	346	9.0	10.7

		SEG		ABSSEG		LYMPH		ABSLYMPH	
Reference	low	0.05		0.22		0.48		2.20	
Reference	high	0.37		4.59		0.93		11.61	
SO2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm Control	warm	0.20	0.19	2.24	1.80	0.74	0.74	8.29	7.06
20 ppm	warm	0.17	0.03	1.62	0.30	0.71	0.07	6.85	0.24
20 ppm Control	cold	0.28	0.11	2.78	1.52	0.64	0.12	6.28	2.10
20 ppm	cold	0.17	0.21	1.52	2.24	0.74	0.72	6.62	7.70

Table B.4 (Cont'd) Hematological values of peripheral blood from steers exposed to 20 ppm of SO_2 (Experiment 3)

		MONOS		ABSMONO		EOS		ABSEOS	
Reference	low	0.00		0.00		0.00		0.00	
Reference	high	0.08		1.03		0.14		1.77	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm Control	warm	0.00	0.01	0.00	0.09	0.05	0.06	0.56	0.52
20 ppm	warm	0.02	0.01	0.17	0.11	0.10	0.07	1.00	0.76
20 ppm Control	cold	0.01	0.00	0.10	0.03	0.07	0.02	0.69	0.24
20 ppm	cold	0.02	0.01	0.18	0.13	0.07	0.06	0.63	0.58

		BASO		ABSBASO		FIBRINOGEN	
Reference	low	0.00		0.00		0.53	
Reference	high	0.01		0.08		1.53	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm Control	warm	0.01	0.01	0.11	0.05	2.00	2.75
20 ppm	warm	0.00	0.01	0.02	0.05	2.25	0.96
20 ppm Control	cold	0.01	0.01	0.05	0.06	1.75	0.50
20 ppm	cold	0.00	0.01	0.00	0.09	1.00	1.25

APPENDIX C

Table C.1 Clinical biochemistry values of peripheral blood from steers exposed to 0, 1, 5 and 20 ppm of SO_2 (Experiment 2)

	ALP		GGT		Glucose		Total protein	
Reference	41 - 116		22 - 64		2.7 - 4.5		65 - 93	
Units	iu/L		iu/L		mmol/L		g/L	
	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	143	48	34	6	4.5	0.23	70	5
Pre-exposure	131	18	31	4	4.6	0.32	67	5
Control 1 ppm	143	64	33	6	4.49	0.23	70	4
1 ppm	136	27	30	4	4.51	0.31	66	4
Control 5 ppm	143	68	33	7	4.16	0.73	70	5
5 ppm	123	33	31	4	4.23	0.42	66	2
Control 20 ppm	148	79	34	8	4.42	0.26	70	4
20 ppm	122	34	30	4	4.35	0.44	65	2

	Albumin		Globulin		BUN		Creatinine	
Reference	30 - 40		34 - 43		1.4 - 8.2		53 - 141	
Units	g/L		g/L		mmol/L		umol/L	
SO2 treatment	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	28	2	42	4	4.5	0.6	117	17
Pre-exposure	28	2	39	3	4.4	0.5	114	13
Control 1 ppm	28	2	43	4	4.6	0.7	112	25
1 ppm	27	1	38	3	4.7	0.5	119	13
Control 5 ppm	28	3	42	4	4.8	0.9	125	14
5 ppm	27	1	39	2	5.0	0.4	128	15
Control 20 ppm	28	2	42	5	4.5	1.0	123	18
20 ppm	28	1	38	2	4.6	0.5	128	14

Table C.1 (Cont'd) Clinical biochemistry values of peripheral blood from steers exposed to 0, 1, 5 and 20 ppm of SO_2 (Experiment 2)

	Osmolality		Anion gap		Sodium		Potassium	
Reference	270 - 300		0 - 20		135 - 153		3.4 - 5.3	
Units	mosm/L		mEg/L		mmol/L		mmol/L	
SO_2 treatment	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	294	9	17	4	142	5	4.3	0.2
Pre-exposure	290	6	17	4	141	3	4.3	0.3
Control								
1 ppm	287	4	15	3	139	2	4.4	0.2
1 ppm	284	5	14	2	138	2	4.4	0.3
Control								
5 ppm	288	4	14	2	139	2	4.5	0.2
5 ppm	286	3	14	1	138	2	4.4	0.3
Control								
20 ppm	288	4	13	1	139	2	4.2	0.2
20 ppm	285	2	13	2	138	1	4.3	0.2

	Chloride		CO2		Calcium		Magnesium	
Reference	88 - 120		28 - 40		1.9 - 2.9		0.7 - 1.23	
Units	mmol/L		mmol/L		mmol/L		mmol/L	
SO_2 treatment	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	102	2	27	1	2.5	0.1	0.85	0.06
Pre-exposure	101	2	27	3	2.5	0.1	0.86	0.05
Control								
1 ppm	100	3	28	2	2.5	0.1	0.81	0.08
1 ppm	99	2	29	1	2.5	0.1	0.85	0.06
Control								
5 ppm	102	3	27	2	2.5	0.1	0.82	0.07
5 ppm	101	2	28	1	2.4	0.1	0.86	0.06
Control								
20 ppm	102	2	29	2	2.4	0.1	0.81	0.1
20 ppm	100	2	29	2	2.4	0.1	0.85	0.07

Table C.1 (Cont'd) Clinical biochemistry values of peripheral blood from steers exposed to 0, 1, 5 and 20 ppm of SO_2 (Experiment 2)

	Phosphorus		Cholesterol		Triglyceride		LDH	
Reference	1.3 - 3		1.8 - 5.1		0.03 - NA		3053 - 5533	
Units	mmol/L		mmol/L				iu/L	
SO_2 treatment	mean	Std. dev	mean	Std. dev	mean	Std. dev	mean	Std. dev
Pre-exposure control	2	0	3	1	0.25	0.03	4194	557
Pre-exposure	3	0	3	1	0.25	0.03	3956	544
Control 1 ppm	2	0	4	1	0.27	0.03	3923	382
1 ppm	2	0	4	6	0.26	0.04	3785	355
Control 5 ppm	2	0	3	1	0.25	0.02	3774	374
5 ppm	3	0	3	1	0.26	0.04	3610	413
Control 20 ppm	2	0	3	1	0.33		3843	556
20 ppm	3	0	3	1	0.27	0.04	3553	458

	Iron		TIBC	
Reference	14 - 27		44 - 82	
Units	umol/L		umol/L	
SO_2 treatment	mean	Std. dev	mean	Std. dev
Pre-exposure control	28	9	78	6
Pre-exposure	34	6	78	8
Control 1 ppm	24	7	73	6
1 ppm	27	6	77	9
Control 5 ppm	26	7	72	9
5 ppm	27	6	73	6
Control 20 ppm	24	6	70	8
20 ppm	28	3	71	5

Table C.2 Clinical biochemistry values of peripheral blood from steers exposed to 0 and 1 ppm of SO_2 (Experiment 3)

		CPK		LDH		AST		ALP	
low	Reference	55		939		62		25	
high		401		1595		98		312	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	146	45	1130	341	59	14	84	25
Pre-exposure	warm	112	26	1078	84	61	5	169	121
Pre-exposure control	cold	163	86	1113	138	60	10	136	76
Pre-exposure	cold	111	13	1163	110	54	8	123	45
1 ppm control	warm	165	50	1219	160	59	12	81	19
1 ppm	warm	110	11	1091	77	62	5	159	106
1 ppm control	cold	233	141	1149	151	69	16	159	134
1 ppm	cold	154	58	1155	53	61	5	130	53
		GGT		TBil		DBil		IBIL	
low	Reference	9		0		0			
high		23		4		1			
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	16	4	2	1	0	0	1	1
Pre-exposure	warm	17	3	2	0	1	1	2	1
Pre-exposure control	cold	18	4	2	1	0	1	1	1
Pre-exposure	cold	16	2	1	1	0	0	1	1
1 ppm control	warm	18	2	1	1	0	0	1	1
1 ppm	warm	17	2	1	1	0	0	1	1
1 ppm control	cold	16	4	1	1	0	0	1	0
1 ppm	cold	17	1	1	0	0	0	1	0

Table C.2 (Cont'd) Clinical biochemistry values of peripheral blood from steers exposed to 0 and 1 ppm of SO_2 (Experiment 3)

		Glucose		Urea		Crea		Ca	
low	Reference	3		7		138		2	
high		6		10		192		3	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	4	2	6	0	114	10	3	0
Pre-exposure	warm	4	0	6	1	109	6	3	0
Pre-exposure control	cold	4	0	7	0	108	23	3	0
Pre-exposure	cold	4	1	6	1	116	23	3	0
1 ppm control	warm	4	0	6	1	106	9	3	0
1 ppm	warm	5	0	6	0	107	8	3	0
1 ppm control	cold	5	0	6	0	101	19	3	0
1 ppm	cold	4	0	6	0	108	7	3	0
		Phos		Mag		TP		ALB	
low	Reference	2		1		72		34	
high		3		1		82		40	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	2	0	1	0	74	3	37	1
Pre-exposure	warm	2	0	1	0	69	2	38	1
Pre-exposure control	cold	2	0	1	0	74	5	37	1
Pre-exposure	cold	2	0	1	0	71	2	36	3
1 ppm control	warm	2	0	1	0	72	3	37	1
1 ppm	warm	3	0	1	0	69	2	38	1
1 ppm control	cold	2	0	1	0	72	3	38	1
1 ppm	cold	2	0	1	0	71	1	37	1

Table C.2 (Cont'd) Clinical biochemistry values of peripheral blood from steers exposed to 0 and 1 ppm of SO_2 (Experiment 3)

		AGR		Chol		Trig	
low	Reference	1		3		0	
high		1		4		0	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	1	0	3	0	0	0
Pre-exposure	warm	1	0	3	1	0	0
Pre-exposure control	cold	1	0	3	0	0	0
Pre-exposure	cold	1	0	3	1	0	0
1 ppm control	warm	1	0	3	0	0	0
1 ppm	warm	1	0	3	1	0	0
1 ppm control	cold	1	0	3	0	0	0
1 ppm	cold	1	0	3	0	0	0
		K		Cl		Na	
low	Reference	4		98		138	
high		5		104		145	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	4	0	98	3	139	2
Pre-exposure	warm	4	0	99	1	139	1
Pre-exposure control	cold	4	0	98	2	139	1
Pre-exposure	cold	4	0	97	2	139	1
1 ppm control	warm	4	0	99	3	138	2
1 ppm	warm	4	0	98	1	138	1
1 ppm control	cold	4	0	100	4	139	1
1 ppm	cold	4	0	99	2	138	0
		TIBC		Iron		IBC	
low	Reference	57		20		37	
high		92		37		55	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Pre-exposure control	warm	67	12	27	10	40	5
Pre-exposure	warm	74	9	31	8	42	15
Pre-exposure control	cold	70	14	25	3	46	12
Pre-exposure	cold	70	14	29	8	40	11
1 ppm control	warm	69	6	30	9	39	6
1 ppm	warm	75	5	29	4	46	6
1 ppm control	cold	72	5	25	4	47	3
1 ppm	cold	71	4	28	3	43	3

Table C.3 Clinical biochemistry values of peripheral blood from steers exposed to 5 ppm of SO_2 (Experiment 3)

		CPK		LDH		AST		ALP	
low	Reference	55		939		62		25	
high		401		1595		98		312	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	163	78	1237	96	60	12	72	21
5 ppm	warm	130	38	1153	78	62	6	149	98
5 ppm control	cold	256	134	1315	232	78	21	153	119
5 ppm	cold	133	18	1204	110	58	6	133	64
		GGT		TBil		DBil		IBIL	
low	Reference	9		0		0			
high		23		4		1			
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	18	2	1	1	0	0	1	0
5 ppm	warm	19	1	2	0	1	0	1	0
5 ppm control	cold	18	4	1	1	0	0	1	0
5 ppm	cold	17	3	1	0	0	0	1	1
		Glucose		Urea		Creat		Ca	
low	Reference	3.1		6.6		138		2.26	
high		6.4		9.6		192		2.64	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	4.5	0.3	5.5	1.1	112	10	2.58	0.12
5 ppm	warm	4.4	0.3	5.9	0.3	115	14	2.56	0.13
5 ppm control	cold	4.6	0.4	6.3	0.3	104	16	2.60	0.09
5 ppm	cold	4.6	0.3	6.3	0.7	112	19	2.65	0.09
		Phos		Mag		TP		ALB	
low	Reference	1.50		0.81		72		34	
high		2.50		1.08		82		40	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	2.39	0.29	0.82	0.05	75	4	36	3
5 ppm	warm	2.43	0.22	0.87	0.06	69	3	38	2
5 ppm control	cold	2.16	0.35	0.76	0.06	73	2	37	1
5 ppm	cold	2.20	0.19	0.90	0.05	70	5	37	2

Table C.3 (Cont'd) Clinical biochemistry values of peripheral blood from steers exposed to 5 ppm of SO_2 (Experiment 3)

		AGR		Chol		Trig	
	Reference						
low		0.80		2.70		0.09	
high		1.05		4.07		0.35	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	0.98	0.19	2.98	0.46	0.25	0.06
5 ppm	warm	1.23	0.16	3.52	0.63	0.25	0.06
5 ppm control	cold	1.06	0.08	2.85	0.47	0.23	0.07
5 ppm	cold	1.13	0.22	3.56	0.82	0.21	0.05
		Na		K		Cl	
	Reference						
low		138		3.5		98	
high		145		4.7		104	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	139	2	4.2	0.3	101	2
5 ppm	warm	140	4	4.2	0.4	101	2
5 ppm control	cold	139	3	4.2	0.3	100	4
5 ppm	cold	139	4	4.2	0.2	101	2
		Iron		IBC		TIBC	
	Reference						
low		20		37		57	
high		37		55		92	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
5 ppm control	warm	24	8	40	6	64	8
5 ppm	warm	26	6	45	7	71	6
5 ppm control	cold	23	4	46	7	69	5
5 ppm	cold	28	8	42	6	70	8

Table C.4 Clinical biochemistry values of peripheral blood from steers exposed to 20 ppm of SO_2 (Experiment 3)

		CPK		LDH		AST		ALP	
low	Reference	55		939		62		25	
high		401		1595		98		312	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	203	139	1300	152	64	16	77	23
20 ppm	warm	146	30	1212	87	67	8	148	99
20 ppm control	cold	233	89	1357	203	76	12	146	102
20 ppm	cold	136	18	1224	74	59	8	132	66
		GGT		TBil		DBil		IBIL	
low	Reference	9		0		0			
high		23		4		1			
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	18	3	2	0	0	0	1	1
20 ppm	warm	19	1	2	1	0	0	2	1
20 ppm control	cold	17	5	2	1	0	0	2	1
20 ppm	cold	18	2	2	1	0	0	1	1
		Glucose		Urea		Crea		Ca	
low	Reference	3.1		6.6		138		2.26	
high		6.4		9.6		192		2.64	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	4.2	0.3	5.9	0.6	108	7	2.6	0.1
20 ppm	warm	4.4	0.2	6.1	0.4	115	15	2.6	0.1
20 ppm control	cold	4.5	0.3	6.4	0.2	104	14	2.6	0.1
20 ppm	cold	4.6	0.2	6.4	0.7	110	16	2.6	0.0
		Phos		Mag		TP		ALB	
low	Reference	1.50		0.81		72		34	
high		2.50		1.08		82		40	
SO2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	2.37	0.24	0.83	0.06	76	4	37	2
20 ppm	warm	2.32	0.31	0.87	0.07	69	1	39	2
20 ppm control	cold	2.31	0.36	0.77	0.10	73	1	38	1
20 ppm	cold	2.21	0.27	0.91	0.06	70	5	37	2

Table C.4 (Cont'd) Clinical biochemistry values of peripheral blood from steers exposed to 20 ppm of SO_2 (Experiment 3)

		AGR		Chol		Trig	
low	Reference	0.80		2.70		0.09	
high		1.05		4.07		0.35	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	0.96	0.16	3.19	0.27	0.26	0.03
20 ppm	warm	1.27	0.16	3.59	0.93	0.26	0.07
20 ppm control	cold	1.12	0.10	3.04	0.41	0.24	0.08
20 ppm	cold	1.17	0.23	3.70	0.93	0.22	0.06
		Na		K		Cl	
low	Reference	138		3.5		98	
high		145		4.7		104	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	138	1	4.1	0.1	100	5
20 ppm	warm	139	2	4.1	0.2	101	3
20 ppm control	cold	138	1	4.0	0.1	99	5
20 ppm	cold	138	2	4.2	0.2	100	2
		Iron		IBC		TIBC	
low	Reference	20		37		57	
high		37		55		92	
SO_2 treatment	Environment	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
20 ppm control	warm	25	4	39	5	64	6
20 ppm	warm	27	5	44	6	71	5
20 ppm control	cold	25	5	44	7	70	5
20 ppm	cold	29	9	43	7	72	7